"It was already one in the morning; the rain pattered dismally against the panes, and my candle was nearly burnt out, when, by the glimmer of the half-extinguished light, I saw the dull yellow eye of the creature open . . ."

- Mary Shelley ❧ *Frankenstein*

THREE STINGS

George got stung by a bee and said,
"I wouldn't have got stung if I'd stayed in bed."
Fred got stung and we heard him roar,
"What am I being punished for?"
Lew got stung and we heard him say,
"I learned somthin' about bees today."

- Shel Silverstein ❧ *Falling Up*

University of Alberta

Transcriptional Regulation of the S-phase Cyclin *CLB5* during Meiotic Development in *Saccharomyces cerevisiae*

by

Sheetal Amritlal Raithatha

A thesis submitted to the Faculty of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of

Doctor of Philosophy ©

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Abstract

The *Saccharomyces cerevisiae* B-type cyclin *CLB5* is the principle S-phase cyclin regulating DNA replication during proliferation and meiotic development. *CLB5* is absolutely essential for successful completion of premeiotic DNA replication. The reason for the essential requirement for *CLB5* during meiotic development is unknown. Reorganization of cell cycle events, including the expression of key genes, may offer a possible explanation. In proliferating cells, the START-specific transcription factor MBF regulates genes whose products are involved in DNA replication, including *CLB5*. MBF is a heterodimeric complex, consisting of the trans-activation subunit Swi6, and the DNA-binding subunit Mbp1. MBF promotes the periodic expression of its target genes by binding to MCB (*MluI* cell cycle box) sequences in their promoters. Even though many of the DNA replication genes expressed in proliferating cells are required for pre-meiotic S-phase, little is known about MBF activity during sporulation. We have discovered that Mbp1 regulates a subset of MCB-containing genes during sporulation, such as *RNR1* and *TMP1*. However, deletion of *MBP1* did not produce any defects in meiotic DNA replication, meiotic recombination or spore formation. Interestingly, we also discovered that Mbp1 does not regulate the expression of *CLB5* during sporulation. Extensive analysis of the *CLB5* promoter revealed that most of the meiotic regulation of this gene is derived from elements within a 180bp region. This region contains a cluster of MCBs and a consensus MSE (Middle Sporulation Element). Surprisingly we

have discovered that MCB-mediated expression of *CLB5* is critical during meiotic development, and this expression is independent of Mbp1. This implicates the existence of another MCB binding factor in sporulating cells. The meiosis-specific transcription factor Ndt80 regulates the expression of middle sporulation genes, including the *CLB*s. Analysis of the reported MSE in the *CLB5* promoter has revealed that this site does not promote the Ndt80-dependent expression of this cyclin. However, we have demonstrated that Ndt80 directly regulates the *CLB5* promoter by binding to a previously unrecognized MSE upstream of the reported potential Ndt80 binding site. Therefore, we reveal *CLB5* to be an intricately regulated gene during meiotic development, and suggest that meiosis-specific mechanisms promote its expression.

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To,

My Loving Parents,
Chandrika and Amritlal Raithatha

Thank You

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List of Terms and Abbreviations

ANK	- domain containing ankyrin repeats
APC	- anaphase promoting complex
ARS	- autonomously replicating sequence
ATP	- adenosine triphosphate
bp	- nucleotide base-pair
BSA	- bovine serum albumin
CDK	- cyclin dependent kinase
cDNA	- reverse-transcribed copies of mRNA
Cki	- CDK inhibitor
codon	- individual unit of the triplicate genetic code encoding a specific amino acid
DAPI	- 4'-6-diamidino-2-phenylindole
dCTP	- 2'-deoxycytidine 5'-triphosphate
DDK	- Dbf4-dependent kinase
diploid	- genetic term indicating 2N DNA content
DNA	- deoxyribonucleic acid
DSB	- double strand break (in dsDNA)
DSC1	- DNA Synthesis Control complex; *S. pombe* MBF
dsDNA	- double-stranded deoxyribonucleic acid
DTT	- dithiothreitol
ECB	- early cell cycle box; Mcm1 binding site
EDTA	- ethylenediaminetetracetic acid
EGTA	- ethylene glycol-bis-(b-aminoethyl-ether)-N,N,N',N'-tetra-acetic acid
EMSA	- electrophoretic mobility shift assay
G_1-phase	- 1st Gap phase in the eukaryotic cell cycle
G_2-phase	- 2nd Gap phase in the eukaryotic cell cycle
gamete	- a haploid cell involved in sexual reproduciton
gametogenesis	- meiotic developmental process whereby diploid germ cells produce haploid gamete progeny
GINS	- Go Ichi Nii San (five one two three in Japanese); a protein complex involved in DNA replication
HA	- haemagglutinin epitope tag
haploid	- genetic term indicating a 1N DNA content
HAT	- histone acetyltransferase
HDAC	- histone deacetylase
heterozygous	- genetic term indicating variable alleles are present at a given locus in a diploid genome
homozygous	- genetic term indicating identical alleles are present at a given locus in a diploid genome
IC	- initiation complex
IgG	- immunoglobulin G
IMAC	- immobilized-metal affinity chromatography
in vitro	- "in glass"; outside the living organism or cell

in vivo	- "in the living"; within a whole organism or cell
Kan^R	- kanamycin resistant
kDa	- kilo Dalton
MI	- meiosis I; 1st (reductional) meiotic nuclear division
MII	- meiosis II; 2nd (equational) meiotic nuclear division
M-phase	- mitotic nuclear division phase
MBF	- MCB-binding factor
MCB	- *MluI* cell cycle box; MBF binding site
MPF	- maturation promoting factor
mRNA	- messenger RNA
MSE	- middle sporulation element
Myc	- c-myc epitope tag
ORC	- origin recognition complex
ORF	- open reading frame
^{32}P	- radioactive phosphorous isotope
PCR	- polymerase chain reaction
pre-RC	- pre-replicative complex
RNA	- ribonucleic acid
RT-PCR	- reverse transcriptase-polymerase chain reaction
SAGA	- Spt-Ada-Gcn5-Acetyltransferase
SBF	- SCB binding factor
SCB	- Swi4/Swi6-dependent cell cycle box
SDS	- sodium dodecyl sulfate
SDS-PAGE	- SDS polyacrylamide gel electrophoresis
S-phase	- DNA synthesis stage
SPM	- sporulation medium
spermatogenesis	- gametogenesis in metazoan males; production of sperm
sporulation	- gametogenesis in *Saccharomyces cerevisiae*
SSC	- saline-sodium citrate
ssDNA	- single-stranded DNA
TCA	- trichloroacetic acid
tetrad	- product of efficient yeast sporulation; an ascus housing four haploid spores
Tris-HCl	- tris (hydroxymethyl) amino methane hydrochloride
UAS	- upstream activating sequence
URS	- upstream regulatory sequence
URS1	- upstream repressor sequence 1, Ume6 binding site
WT	- wild type; designation for yeast strains that contain no mutations – note, WT strains contain auxotrophic mutations for genetic manipulation.
YEPD	- yeast extract/peptone/dextrose; rich medium supplemented with dextrose for yeast propagation
YEPGlyc	- yeast extract/peptone/glycerol; rich medium supplemented with glycerol for yeast propagation
YEPKAc	- yeast extract/peptone/potassium acetate; rich medium supplemented with potassium acetate for yeast propagation

Chapter I

Introduction:

A Review of the Eukaryotic Cell Cycle and Meiotic Development in the Budding Yeast *Saccharomyces cerevisiae*

I.1 – The Cell Cycle

"*Omnis cellula e cellula*" (All cells from cells) – Rudolf Virchow (1821-1902)

I.1.a. - Leaps and Bounds: The Pursuit of Cell Cycle Control

In 1665, English physicist Robert Hooke made a pivotal discovery. Using a compound microscope to examine slices of cork, he observed an array of tiny pores, which he termed "cells". Published as "Observation XVIII" in his famed book, *Micrographia*, Robert Hooke's discovery would stand as the first line of evidence for the basic unit of life – the cell (141). In the decades to follow, advances in microbiology led to the proposal of a unified "Cell Theory". German physician Matthias Jacob Schleiden and German biologist Theodore Schwann demonstrated that the nucleated cell is the basic structural and functional unit in plants and animals (374). Having garnered much more attention, biologists began contemplating the nature of the cell. In 1855, German physician Rudolf Virchow made a striking revelation: "Where a cell arises, there a cell must have previously existed (*omnis cellula e cellula*), just as an animal can spring an animal, a plant only from a plant" (367). With this observation, Virchow had illuminated the process of cell division, the fundamental manner in which living organisms propagate.

During the second half of the nineteenth century, rapid developments in microscopic techniques allowed for the finer observation of the cell division. More accurate descriptions of the basic steps of mitosis were outlined. In 1882, German anatomist Walther Flemming observed in cell a thread-like substance he termed chromatin (258, 280). The use of aniline dyes to stain chromatin allowed Flemming to follow the process of cell division in far greater detail. He called this process "mitosis" from the Greek word for "thread". Flemming's significant observation was of the most apparent event to occur during mitosis, the segregation of chromosomes. In 1888, observations made by Theodore Boveri regarding the continuity of chromosomes during cell division allowed him to suggest that chromosomes were involved in heredity (280). And in 1910, Thomas Hunt Morgan established the chromosomal theory of heredity, and the link between chromosomes and inheritance would become more widely accepted (258).

Investigations offering further insight into how cell division was controlled became the domain of experimental embryologists in the early 1900's. Through careful observation and inventive manipulation of developing early embryos, a number of theories emerged postulating possible mechanisms controlling how cells divide. For instance, in 1903 Richard Hertwig's studies allowed him to formulate the theory of the "karyoplasmic ratio" (258). This theory suggests that a fixed proportion exists between the nuclear and cytoplasmic masses. In essence, an increase in the cytoplasmic mass of a cell would unbalance this ratio leading to karyoplasmic tension. Hence, cell division ensues to re-establish the nuclear/cytoplasmic relationship. Illuminating experiments done on the protozoan *Amoeba proteus* by Hartmann in 1928 (258), and again repeated by Prescott in the mid 1950's (289-291), provided further evidence for the karyoplasmic ratio theory. Both these zoologists performed repeated microdissections on growing amoebae, in which portions of the cytoplasm were removed, resulting in a smaller cytoplasmic mass with respect to the remaining nucleus. These experiments demonstrated a delay in cellular division, presumably due to the fact that these cells required further growth to attain the cytoplasmic mass necessary for division to proceed. An important principle underlies these experiments with regards to cell cycle control - mitosis can be regulated by properties of the cytoplasm. Specifically, this work suggested that a factor, whose activity promotes nuclear division, might be accumulating proportionally to the cytoplasmic mass in cells. Work done by Erik Zeuthen on the ciliate *Tetrahymena* implicated this division factor to be a protein (396). Zeuthen demonstrated a transition point in the cell division cycle by using high temperature or pressure treatment on these cells. When cells were treated after this transition point, nuclear division continued. However, treatment of cells before this transition point delayed the onset of cell division, resulting in a population synchronized to the same point in the cell cycle. These cells required a fixed interval of time to recover from the treatment, indicating that a particular process needed to be repeated to prepare for mitosis. Interestingly, similar delays in the onset of mitosis were also seen in cells treated with pulses of the protein synthesis inhibitor cycloheximide. This led Zeuthen to suggest that before the onset of the transition point, cells accumulate proteins which create specific complexes required for cell division to occur. Shortly after Zeuthen's findings in Tetrahymena, a similar transition point was observed by Howard Temin in chicken cells (345). Temin demonstrated that stationary chicken fibroblasts, when stimulated to enter cell division by calf serum, eventually progressed independently of external mitogenic signals. This implicated an important transition, where these cells become committed to a round of DNA replication and mitosis during G_1-phase. This transition was characterized further by Arthur Pardee, who introduced the term "Restriction Point" to define this commitment step (270). Then in 1985, Anders Zetterberg and Olle Larsson used time-lapse video recordings of mammalian cells in culture, which had been subjected to short periods of serum starvation, to determine the precise location of the restriction point relative to the initiation of S-phase (395). Interestingly, it was also shown that inhibition of protein synthesis with cycloheximide mimicked serum starvation in these cells – G_1-phase cells before the restriction point halted mitotic progression, while cells that had passed the restriction point continue through the cell cycle.

Taken together, work done up to the 1980's, using microscopic manipulation and observation of a number of microbial organisms and cultured cells provided significant insight into the underlying principle of cell cycle control. Essentially, it was believed that the accumulation of division proteins to levels required for creating protein complexes was necessary to initiate cell division. These division proteins may be synthesized at a rate relative to the increase in cytoplasmic mass. And since the invariability of the karyoplasmic ratio implicates a fixed relationship between the mass of the cytoplasm and the DNA content within the nucleus, it was suggested that these division proteins may actually be acting upon nuclear components. This would bring about changes within the nucleus, prompting DNA replication, mitosis and cellular division.

Though it was widely accepted that division proteins were responsible for initiating the transition into cellular division, the nature of these proteins and the structures they were presumed to form remained completely unknown. How these factors influenced the regulation of DNA replication and the mitotic division of the nucleus became central to the pursuits of investigators in cell cycle control. The greater advancements of molecular biology in the late 1970s and during the 1980s allowed cell biologists, geneticists and biochemists to combine their efforts and unravel the greater workings of the cell cycle in molecular terms.

As Hertwig defined the nucleocytoplasmic relationship central to his karyoplasmic ratio theory, he was also careful to point out some exceptions to this homeostatic rule. For example, the oocyte underg[illegible] increased cellular growth, without undergoing nuclear division, greatly increasing the cytoplasmic mass relative to the nucleus (258). Other unique characteristics of oocytes were also recognized around the turn of the 20th century. It was discovered that immature oocytes remain in an arrested state, some being stalled just prior to meiotic M-phase (258). Also, it was observed that isolated amphibian oocytes could be matured by the addition of pituitary extracts, prompting these oocytes to enter M-phase. Embryologists, such as Yoshio Masui, took advantage of these large cells for microsurgical techniques to study cellular and developmental processes, making significant contributions to the molecular mechanisms of cell cycle control. Masui, further investigating the maturation promoting activity of pituitary extracts, demonstrated that oocytes from *Rana pipiens* prepared free of surrounding follicular cells, were responsive to the hormone progesterone (218). These progesterone treated oocytes underwent processes which closely resembled that of mitotically dividing cells: nuclear envelope breakdown, chromosomal condensation, and preparations for chromosomal divisions. This system, in effect, emerged as a convenient experimental system supporting investigations into M-phase inducing factors.

Inspired by the strongly supported view that cytoplasmic factors controlled nuclear activities, increasingly favored by embryologists of the time, Masui and his colleagues demonstrated that cytoplasm taken from progesterone-treated *Rana* oocytes was able to stimulate maturation when injected into untreated eggs (219). Therefore, a putative factor in the cytoplasm of maturing oocytes, called the "Maturation Promoting Factor" (MPF) induced progression into M-phase in an arrested immature oocyte.

Further experiments involving the microinjection of a fixed quantity of cytoplasm, extracted from maturing oocytes at various times post progesterone treatment, demonstrated that MPF activity appeared 6 hours after induction of maturation, and 3 hours before nuclear envelope breakdown. Another key contribution to the developing field of cell cycle control research offered by Masui and his colleagues was their pioneering use of cell-free egg extracts to examine MPF activity and mitosis *in vitro* (199-201). Amazingly, when sperm nuclei were added to extracts derived from activated *Rana* eggs, they initially formed a pro-nucleus, followed by chromosome condensation and a continued procession through the cell cycle undergoing mitosis.

The later extension of this procedure by Lohka, Hayes and Maller, developing a system based on *Xenopus* egg extracts, offered a favorable assay system for the biochemical isolation of the MPF (198). Through a multi-step preparation MPF was purified >3000-fold. Fractions retaining the highest MPF activity were found to consist of two proteins with molecular masses of 32 kDa and 45 kDa, clearly demonstrating the source of MPF activity to indeed be protein factors in the cytoplasm.

Parallel to the work done by embryologists on maturing oocytes, geneticists also made critical advancements to the understanding of cell division. Contrary to the embryologist's decree of cytoplasmic control over the nucleus, geneticists were of the belief that genetic messages originating from the nucleus imparted regulatory influence on the cytoplasm. During the early 1970s many of the cellular events and some biochemical processes had been described in significant detail, however, the genes that influence the orderly progression of the cell cycle and the nature of the proteins they encode were mostly unknown. The animals of choice for the geneticist were the single-celled eukaryotes, budding yeast and fission yeast. These model organisms offered geneticists a powerful tool to study genetic mutation and its effects on many cellular processes, including the control of cell division. Pivotal work done by Leland Hartwell during this period led to the discovery and identification of the central players involved in the progression of the cell cycle.

In a series of experiments, Hartwell isolated a comprehensive collection of cell division cycle (CDC) mutants in the budding yeast, *Saccharomyces cerevisiae* (117, 118, 121). Through the creation of strains carrying temperature-sensitive mutations he was able to analyze those genes which perform essential cell cycle functions by characterizing the yeast mutants blocked at specific stages of cell division. Characterization of the phenotypes of these CDC mutants allowed Hartwell to define the major cell cycle transitions in budding yeast as well as the major control points in the cell division cycle which he termed as "checkpoints" (120). These checkpoints illuminated the intricacies of cell cycle control, specifically the dependence of later events on the successful completion of earlier events in cell division.

A specific example of this is the requirement to complete DNA replication before mitosis. Incomplete DNA replication or DNA damage instigates checkpoint responses leading to a block prior to the onset of M-phase. This cell cycle block is not relieved until the respective defect has been corrected. In essence, these checkpoints are activated signal-transduction pathways that initiate inhibitory signals as a response to problems arising in upstream processes. Another important checkpoint, termed "START", actually defined the first major transition initiating the sequential cell cycle events. This point, which responds to nutritional, hormonal and cell-size conditions, was exemplified by the function of one gene in particular, *CDC28* (133). The activation of two independent pathways, one leading to bud emergence and cytokinesis, and the other leading to DNA replication and nuclear division, requires *CDC28*, proving this gene to be a central regulator of the budding yeast cell cycle.

Inspired by Hartwell's work in *S. cerevisiae*, Paul Nurse used the same genetic approach to characterize CDC mutants in the fission yeast *Schizosaccharomyces pombe* (256, 262). He initially began by describing mutants which were able to advance cells into mitosis before they had reached the appropriate cell size. These so called *wee* mutants represented critical regulators of the cell cycle, and one in particular was identified as a central cell cycle control gene in *S. pombe*, *cdc2*. This gene became a major focus due to the fact that it was critical for mitosis (261). Like *CDC28* in budding yeast, *cdc2* seemed to coordinate growth to cellular division, however there were major differences in their apparent functions. *CDC28* proved essential in the first Gap phase prior to DNA replication, G_1 (133), whereas *cdc2* was essential in the second Gap phase before the mitotic division, G_2. Some exceptions to these observations fostered speculation of a possible relationship between these two "master regulators". An important need for *cdc2* in G_1 was determined as fission yeast cells emerge from stationary phase (259) and one allele of *CDC28* reportedly arrested budding yeast cells in G_2 (285). And so, together with David Beach, Nurse demonstrated that *S. pombe cdc2* is, in fact, the functional homologue of *S. cerevisiae CDC28* (15).

It was then that a flurry of work ensued, leading to an amazing succession of unifying discoveries, demonstrating that the *S. cerevisiae CDC28* gene product, the *S. pombe cdc2* gene product, and the MPF activity in *Xenopus* were related 34 kDa serine-threonine protein kinases (257). These 34 kDa protein kinases (p34) were postulated to be central regulators of the cell cycle, driving both DNA replication and chromosomal segregation. Though this discovery spawned the notion of a conserved mechanism underlying the division of eukaryotic cells, it was still not understood how a single enzyme could promote two separate stages in cell division.

In the early 80's, Tim Hunt began studying protein synthesis in marine invertebrates. One simple biochemical experiment, in particular, would lead to the discovery of the most fundamental concept of cell cycle regulation. By analyzing the pattern of protein synthesis following the fertilization of sea urchin eggs, he revealed a striking pattern: one of the most highly abundant proteins at early times abruptly disappeard upon division of the fertilized egg, and then reappeared periodically there afterwards (86). He named this highly abundant protein "cyclin". He compared his findings to work done on measuring MPF levels in maturing oocytes, where subsequent activations of MPF activity required protein synthesis (103), and wondered whether he had stumbled upon a possible mechanism for how cell cycle progression is regulated: are cycles of protein synthesis and proteolysis the basis for driving the events of cell division? Further experiments clearly demonstrated that the cyclin protein disappeared moments before cytokinesis and reappeared soon afterwards. These waves of cyclin synthesis interspersed by sudden proteolysis would prove to be the driving force behind the cell cycle (239), and cyclins would be identified as the 45 kDa component of MPF (183). Hence cyclins became known as the switch activating the p34 kinase, allowing these "cyclin dependent kinases" (CDKs) to target specific substrates in the cell. The coordinated, cyclical expression of cyclins would adjust the activity of their CDK partners, producing temporal modifications of substrates at the times they are required to promote specific functions in the dividing cell. In this way, cyclins endow CDKs with the ability to act as master regulators for the various stages of the cell cycle.

From microscopic work done at the turn of the century describing the cytology of dividing cells, to the present day pursuits of genetics, biochemistry and microbiology unraveling the mechanisms controlling cellular proliferation, the cell cycle continues to be the subject of intense investigation. Understanding this highly regulated program leading to the reproduction of a living cell, and the intricate complexities crucial to this process has benefited from work done on a surprisingly wide range of organisms from the single celled budding yeast, to higher order eukaryotes such as frogs, sea urchins and mice. The recognition that the basic processes and regulated mechanisms within the cell cycle are universally conserved among eukaryotes was one of the greatest breakthroughs of this era.

I.1.b. - Fundamentals of the Budding Yeast Cell Cycle

In the early 1970's, the sequential stages of the eukaryotic cell cycle were characterized (236). Events of the nucleus, such as DNA replication and chromosomal divisions, and periods of cytoplasmic activity and growth were described. Essentially, the basic model of the eukaryotic cell cycle has been divided into four stages; these being DNA Synthesis and Mitosis, both separated by intervening Gap phases (4, 147). Briefly, cells begin the division cycle in a growth stage termed Gap1 (G_1) phase. Here cells monitor their environment, as well as their growth, committing to enter a cell division cycle only when conditions are favorable. S-phase, where DNA synthesis occurs, follows G_1. This stage involves the faithful duplication of chromosomes, resulting in paired identical sister chromatids. After S-phase, a second Gap phase ensues (G_2), where cells ensure the successful completion of DNA replication before proceeding into the nuclear divisions, or M-phase (Mitosis). M-phase can be subdivided into distinct stages based on the events observed during the separation of the chromosomes: prophase, metaphase, anaphase and telophase. During prophase, in most eukaryotic cells, the nuclear envelope breaks down and the genetic material condenses into visible chromosomes, composed of paired sister chromatids. The microtubules of the cell also reorganize forming mitotic spindles, which make attachments to the chromosomes. During metaphase, the individual chromosomes are aligned by the mitotic spindle, whereupon they remain poised for segregation. The separation of sister chromatids marks the onset of anaphase, resulting in the movement of the separated chromatids towards opposite spindle poles. Finally, during telophase, chromosomes decondense, nuclei reform, and the cell proceeds to divide. Most eukaryotic cells conform to this standard view of the cell cycle, demonstrating that highly conserved mechanisms control its regulation. As such, many advances in our knowledge of the cell cycle have been attributed to studies focusing on lower order eukaryotes such as the budding yeast *Saccharomyces cerevisiae* (as described above).

S. cerevisiae represent a simple eukaryotic organism. Unlike metazoans, which contain a collection of cyclins and CDKs regulating different aspects of the cell cycle, budding yeast express a single CDK (Cdc28) solely for regulating this process. And as such, nine coordinately expressed cyclins regulate this single CDK promoting cell division. These nine cyclins include three G_1-cyclins (Cln1, Cln2 and Cln3) and six B-type cyclins (Clb1, Clb2, Clb3, Clb4, Clb5 and Clb6). Indeed, *S. cerevisiae* continues to be a pivotal model organism used in cell cycle research, and happens to be the central focus of the graduate work presented hereafter. This section will continue with a brief outline of the budding yeast cell cycle, with emphasis on those aspects most relevant to the subject of this thesis.

The START Transition: Cells respond to various environmental, metabolic and genetic cues to determine when and if to begin a cell cycle. Budding yeast have developed intricate mechanisms to coordinate cell growth and environmental factors to cell division. The capacity for budding yeast to sense their environmental surroundings is critical to ensure that enough resources are available to undergo the energetically costly cell division process. Additionally, all cells must ensure that they are metabolically ready to initiate a cell cycle. When all conditions are met, cells can commit to a cell cycle. This commitment step in yeast is termed "START" (158). Equivalent to the restriction point in higher eukaryotes, START initiates mechanisms which promote the transition from G_1 to S-phase.

In budding yeast, The G1-cyclin Cln3 has proven to be a key regulator at START (58, 69, 175, 243, 341, 355). Through its activation of Cdc28, Cln3 promotes passage through START by initiating a wave of transcription necessary to drive the G_1/S transition. Though Cln3 accumulates steadily throughout the cell cycle, this cyclin is a relatively low abundance protein, and is constitutively unstable (59, 105, 355). This would indicate that Cln3 levels are primarily dependent on its rate of synthesis. The rate of Cln3 synthesis has actually been demonstrated to coordinate with the growth rate of the cell (98). Upon its protein expression, this cyclin has been shown to localize to the nucleus (80, 231). As growth ensues in G_1, Cln3 expression continues, and as a result, the effective concentration of this cyclin within the nucleus rises. It is believed that a threshold level is eventually reached, at which time Cln3-Cdc28 activity can promote START. Mechanisms such as transcriptional regulation, nutritional signaling, translational regulation, and specific inhibition of its activity all coordinate to regulate Cln3 function (3, 100, 101, 114, 126, 250, 273, 278, 287, 354).

The G_1/S Transition: Large scale array analysis in budding yeast has revealed that the wave of transcription initiated by Cln3-Cdc28 in late-G_1 leads to the expression of >200 genes (44, 334). Many of these genes have been shown to participate in cell cycle-related events. Two primary regulators of this late-G_1 expression are the transcription factors SBF (SCB Binding Factor) and MBF (MCB Binding Factor) (228). These START-specific transcription factors bind to specific *cis*-regulatory elements in the promoters of their target genes activating their expression. Roughly, SBF targets are involved in spindle-pole body duplication, bud emergence and cell wall synthesis, while MBF targets mostly constitute those genes involved in the initiation and regulation of DNA replication (16, 28, 149, 150, 174, 316).

Both SBF and MBF are heterodimeric complexes, sharing related characteristic features. The SBF is a complex of Swi4 (DNA binding subunit) and Swi6 (trans-activation subunit) (248), and it specifically recognizes a promoter element termed SCB (Swi4/6-dependent cell-cycle box) (7, 8, 30). The MBF shares the same trans-activation subunit, Swi6, however employs a different yet homologous DNA-binding subunit, Mbp1 (174). MBF also shows specificity for a different regulatory element named MCB (*MluI* cell-cycle box).

Though the exact mechanism whereby these two transcription factors promote the expression of their target genes remains unclear, the transcriptional activity of SBF and MBF is essential for the G1/S transition. This is supported by the observations that *swi4 swi6* and *swi4 mbp1* mutants arrest prior to START and are inviable (31, 174, 248). Though both these transcription factors share Swi6, null mutations of *swi4* actually display the greatest phenotypic defects. *swi4* cells are viable but exhibit slower growth, producing a large-cell misshapen phenotype (8, 31, 335). *swi6* mutants also develop large cells, however their phenotype is surprisingly not as severe as those seen in *swi4* strains (31). In contrast to these mutants, *mbp1* cells show no obvious growth defects, specifically displaying timely bud emergence and initiation of S-phase (174). *mbp1 swi6* strains are also viable, resembling the phenotype seen in *swi6* cells. Of added interest is the observation that overexpression of *SWI4* suppresses the defects seen in the *swi6* background (31), however the reverse does not hold true (8). Though it remains unclear what these genetic interactions specifically suggest, it is reasonable to conclude that intricate mechanisms, possibly involving co-regulatory factors, influence the activity of SBF and MBF.

An added complexity to the nature of these related transcription factors further obscures our understanding of their exact activities. Even though optimal binding sites determined for SBF and MBF prove to be distinct, considerable overlap in their specificities exists (174, 272). Using *in vitro* assays it was shown that Swi4 can bind to MCB motifs and Mpb1 can bind SCB motifs. Transcription of a reporter driven by multiple synthetic MCBs in tandem is reduced 2 to 8 fold in a *swi4* background (364). SBF-mediated transcription of the G_1-cyclin *CLN1* has been shown to rely on MCB elements in its promoter (272). Also, results obtained from global analysis of SBF and MBF genomic binding-sites suggest putative targets include overlapping sets of genes (155, 324). This potential partial redundancy in their activities may explain some of the genetic data presented above.

Despite the apparent structural similarity and related roles in promoting late-G_1 transcription, evidence exists to suggest that SBF and MBF may be functioning differently to promote the periodic expression of their respective targets. At those genes solely reliant on SBF for their expression, it has been observed that this transcription factor is required for full expression and periodicity of these targets (29, 30). In contrast, those genes that are regulated by MBF are reported to maintain a strong basal expression throughout the cell cycle, requiring this transcription factor solely for their periodic expression (174).

Specifically, it is believed that the MBF acts to promote transcription during G_1/S, and represses transcription during other stages (174).

Even though it is not known exactly how SBF and MBF coordinate to promote late-G_1 gene expression, one aspect remains clear - the activation of these transcription factors is absolutely dependent on Cln3-Cdc28 activity (104, 213, 321, 341, 356). The mechanism by which Cln3-Cdc28 promotes SBF and MBF activity has been somewhat elusive. However, recent work done to seek out additional factors regulating G_1-specific transcription has demonstrated a promising mechanism for SBF activation by Cln3. The protein Whi5 associates with the SBF and acts as a repressor of SBF transcriptional activity (53, 63). Whi5 contains a CDK consensus phosphorylation sequence, and can be phosphorylated by Cln3-Cdc28 *in vitro* (53, 63). This phosphorylation disrupts the association between Whi5 and the SBF. Therefore, a straightforward model was proposed delineating the mechanism of SBF activation. Whi5 binds to SBF repressing its transcriptional activity in G_1. Upon passage through start, activated Cln3-Cdc28 phosphorylates Whi5 promoting its dissociation from SBF. Thus, SBF is free to promote the expression of its target genes. Whi5 also demonstrates the ability to bind to the MBF (53), however the transcription of MBF-dependent genes remains predominantly independent of this repressor protein (63). Though Whi5 does not seem to influence MBF activity, another repressor may exist for this transcription factor, mirroring the regulation observed for SBF. The discovery of this mechanism activating the SBF encouraged the investigators to propose an analogy to the regulation of the metazoan G_1/S transcription factor E2F. The retinoblastoma tumor suppressor protein Rb represses the transcriptional activity of E2F (336). Initiation of the G_1/S phase transition in mammalian cells relies on the phosphorylation of Rb by cyclin D-Cdk4/Cdk6 and cyclin E-Cdk2. This serves as one of many mechanisms influencing the activity of E2F, but provides an exciting parallel between mammalian cells and yeast. It is prudent to note that no protein homology exists between Whi5 and Rb, however the mechanism may be a conserved strategy.

Of the many genes that are expressed during the late-G_1 transcriptional wave, four genes in particular prove to be central in regulating this progression. These are the SBF targets *CLN1* and *CLN2* (381), and the MBF targets *CLB5* and *CLB6* (180, 316). *CLB5* and *CLB6* are the first pair of B-type cyclins to be expressed during the budding yeast cell cycle, and are classified as the principle cyclins involved in regulating DNA replication (83, 180, 316) (see "DNA Replication" below). Once expressed, the activities of Clb5-Cdc28 and Clb6-Cdc28 during G_1 are kept in check by the CDK inhibitor (CKi) Sic1 (227, 315). In metazoans, CKis have been well documented to play roles in regulating processes such as cell division and development, and to act as tumor suppressors (320).

Sic1 shows no noticeable homology to mammalian CKis, however it does bear some functional resemblance. Sic1 is able to inhibit Clb-Cdc28 activity by binding specifically to these complexes and excluding substrates from the kinase active site (363). *SIC1* expression begins in late-M/early-G_1, is predominantly abundant during G_1, and abruptly disappears upon progression into S-phase (73, 229, 315). Though Sic1 is not essential, *sic1* cells demonstrate a higher incidence of genetic instability, resulting in a significant percentage of cells becoming arrested in G_2 (73, 255). This arrest is attributed to premature initiation of DNA replication seen in *sic1* mutants due to unregulated Clb-Cdc28 activity in G_1, which is believed to cause DNA damage and chromosome loss (315). Therefore, to promote the timely initiation of S-phase, Sic1 is required to inhibit Clb5/6-Cdc28 activity until the cells have completely prepared to replicate their DNA (315).

Sic1 inhibition is relieved by the activity of the G_1-cyclins Cln1 and Cln2 (313, 362). In fact, the relief of Sic1 inhibition on Clb-Cdc28 activity proves to be the only essential function of the Clns at START. Inactivation of *sic1* suppresses the G_1 defect seen in *cln1 cln2 cln3* mutant cells, allowing these cells to progress through to S-phase (84, 313, 353). Cln-Cdc28 activity relieves Sic1 inhibition by directly phosphorylating Sic1 on multiple CDK consensus sites, promoting Sic1 degradation by the 26S proteosome (87, 242, 326, 362). This degradation is mediated by the ubiquitin ligase SCF^{Cdc4} (87, 363). Phosphorylated Sic1 is recognized and bound by the SCF^{Cdc4} and subsequently ubiquitinated, flagging it for degradation.

Ubiquitin-dependent proteolysis is a highly conserved mechanism involved in a number of cellular processes, including transcriptional regulation, protein trafficking, and the cell cycle, to name a few (134). This process requires the coordinated effort of three different enzymes/enzyme complexes: an ubiquitin-activating enzyme (E1), an ubiquitin-conjugating enzyme (E2) and an ubiquitin ligase (E3). E1 enzymes use an ATP-dependent mechanism to activate ubiquitin (Ub) by forming a high-energy thioester bond between an active-site cysteine and the C-terminus of Ub. Once activated, the Ub is transferred to an E2 via a transthiolation reaction. This Ub-bearing E2, together with the E3, covalently attaches the Ub to a lysine residue on a target protein destined to be destroyed. Multiubiquitin chains are then created by these same enzymes, producing a signal promoting the degradation of this protein by a large, multisubunit complex of proteases called the 26S proteasome.

Sic1 contains nine CDK consensus sites, and it has been reported that mutants lacking four of these sites (Sic1-Δ4P) are no longer degraded (362). In fact, overexpression of Sic1-Δ4P arrests cells in late G_1 due to the persistent inhibition of Clb-Cdc28 activity. To further elucidate the properties of Sic1 phosphorylation, experiments were done involving the reintroduction of CDK phosphorylation sites into a mutant Sic1 lacking all nine sites (242). It was determined that at least six of these CDK consensus sites need to be phosphorylated for efficient degradation of Sic1 at G_1/S. This seemed to suggest that multi-site phosphorylation may function as a mechanism to sense a threshold for Cln-Cdc28 activity. Basically, at low Cln-Cdc28 levels Sic1 remains stable even if a few sites have been modified, and as Cln-Cdc28 levels rise, Sic1 becomes more highly phosphorylated, thereby leading to its destruction and the timely initiation of S-phase.

The G_1/S transition is a highly controlled process in the eukaryotic cell cycle, involving a network of regulatory interactions. Figure I-1 presents a model outlining the key regulators and the basic interactions involved in the G_1/S transition in S. *cerevisiae*.

DNA Replication: During S-phase, eukaryotic chromosomes are replicated in a bi-directional manner via replication forks that originate from multiple sites along each chromosome. To maintain genetic integrity and cell ploidy, initiation of S-phase must occur in a timely fashion and only once per cell division cycle, and thus, the regulation of DNA replication must be tied into the central cell cycle control machinery. The regulation of DNA replication has been generalized as a two-step process (349). The first step involves the formation of multi-protein complexes at sites of replication initiation called Pre-Replicative Complexes (pre-RC). This step occurs during Mitotic exit and early-G_1. The second step occurs at START, where activated kinases trigger the onset of replication by modifying specific targets within the pre-RC and promoting further interactions of key factors required for DNA synthesis.

The specific sites along chromosomes where pre-RCs form are termed origins of replication (337). These are short DNA sequences (replicator sequences) that are targeted by components of the pre-RC, creating nucleation sites for the assembly of higher order complexes that can eventually promote the initiation of DNA replication (32, 65, 142, 251). In budding yeast these origin sequences are termed Autonomously Replicating Sequences (ARS elements) (251). An important protein complex that acts to nucleate the formation of the pre-RC and is believed to remain constitutively bound to the ARS throughout the cell cycle is the Origin Recognition Complex (ORC) (18, 66, 67, 295, 301). ORC is a six-subunit complex composed of the proteins Orc1, Orc2, Orc3, Orc4, Orc5 and Orc6. The ORC has been shown to bind and hydrolyze ATP, an activity which is believed to be important during the initiation of DNA synthesis (11, 43).

As mentioned above, formation of the pre-RC occurs during late M/early-G_1 through a process termed "origin licensing" (45, 302). Origin licensing involves the further addition of replication factors to ORC at origins, and coincidently, many of these factors are expressed at this stage of the cell cycle (324). Cdc6, a member of the AAA+ family of ATPases is crucial component of the pre-RC (10). It has been shown, as with ORC, that ATP-binding and hydrolysis play key roles in Cdc6 function regulating DNA replication. Cdt1 is another protein of the pre-RC originally identified in *Schizosaccharomyces pombe* (136, 254). This protein is needed with Cdc6 to cooperatively recruit the MCM complex to origins as the last step in licensing.

The MCM complex is a hetero-hexameric assembly composed of the proteins Mcm2, Mcm3, Mcm4, Mcm5, Mcm6 and Mcm7 (78). Mcm2-7 are highly related proteins, however they are distinct enough that each represents a subclass of Mcms conserved across all eukaryotes. This would suggest that a highly important role exists for each Mcm subunit. Indeed, deleting any individual *MCM* leads to lethality in both *S. cerevisiae* and *S. pombe* (78, 167). The hexameric complex formed by the Mcm proteins produces a doughnut-like toroidal structure containing a central cavity (2, 310). Experiments suggest that upon MCM loading to the ARS, DNA is passed through the central cavity. *In vivo* studies have demonstrated a role for the MCM complex at both origins and the replication fork, and it is believed that the MCM acts as a DNA helicase, unwinding the DNA duplex at origins and during progression of the replication fork (185, 197, 379, 400). Biochemical data has confirmed that MCM proteins have DNA helicase activity *in vitro* (153, 188, 189, 389).

The presence of an ARS sequence alone does not necessarily constitute an origin of replication. Local chromatin structure also plays a significant role in origin selection and timing of origin firing. For instance, the positioning of a nucleosome over a plasmid-borne ARS1 element from *S. cerevisiae* prevents the replication of the plasmid in cells (325, 347). Also, the timing of origin firing has been attributed to the context of the origin along a chromosome (ie. many late origins have been associated with heterochromatic regions) (68) further demonstrating the importance of chromatin structure on regulation of DNA replication.

The transition from the pre-RC to the Inititation Complex (IC) promoting the initiation of DNA replication is characterized as "origin firing". This activation of the pre-RC involves the association of other proteins and complexes to the origins, along with the targeted activity of kinases which play essential roles in regulating this transition. These kinases are the B-type cyclin-CDKs as well as the Dbf4 dependent kinase (DDK). Origin firing is dependent on the passage through START, at which point these kinases become active (349). Though the requirement for these kinases is undeniable, the exact mechanism by which they promote DNA replication remains unclear.

As described above, Clb5/6-Cdc28 kinases are activated via the destruction of Sic1 at START. These B-type cyclins promote the initiation of DNA replication and function as the primary S-phase cyclins in budding yeast (83, 180, 316). It has been demonstrated that in *clb5* mutants, DNA replication initiates in a timely manner, however the duration of S-phase is distinctly extended compared to wild type cells. In *clb6* mutants, S-phase appears normal, suggesting that *CLB5* expression is sufficient for these cells to promote efficient DNA replication. It has been suggested that Clb5-Cdc28 activity has the ability to activate both early and late origins of replication (a classification based on the relative timing of origin firing during S-phase), and Clb6-Cdc28 can only activate early origins (72). This can explain why *clb5* cells demonstrate an extended S-phase - if only early origins have fired, then DNA synthesis through late origins would depend on passive replication through these regions. However, recently, it was shown that Clb5 and Clb6 display different protein stabilities (156). Clb5 persists throughout S-phase, and is degraded during mitosis. In contrast, Clb6 is degraded early during the G_1/S transition, only appearing for a short period of time. Interestingly the expression of hyperstabilized Clb6 can rescue the replication defect seen in *clb5* mutants (156). This suggests that, rather than functional differences, reduced Clb-Cdc28 activity produces the extended S-phase in *clb5* cells. Though Clb5 and Clb6 are the primary cyclins regulating mitotic S-phase, their activity is not essential for this process. *clb5 clb6* double mutants display a noticeable delay in S-phase initiation essentially producing an elongated G_1 phase (316). However, it is interesting to note that once launched, the duration of S-phase in these double mutants appears similar to wild-type cells. A significant degree of functional redundancy is observed among the B-type cyclins in *S. cerevisiae*, and it is this property that promotes the eventual activation of S-phase in *clb5 clb6* cells. (315). This is clearly demonstrated by the inhibition of DNA replication seen in a strain lacking all six *CLB* cyclin genes.

The other kinase important for the regulation of DNA replication is the DDK. This kinase is comprised of the Dbf4 regulatory subunit and the Cdc7 kinase (25, 42, 265). DDK is functionally similar to a cyclin-CDK kinase, in that regulation of DDK is dependent on the G_1 expression of *DBF4*. DDK activity is essential for replication, as demonstrated by the observation that *cdc7* mutants arrest in late G_1, after START and immediately before S-phase (139). Evidence suggests that the main and possibly sole purpose that the DDK serves is to phosphorylate Mcm2, thereby resulting in a conformational change of the MCM complex, possibly leading to local melting of the origin DNA (34, 115, 157, 168, 190, 217, 265, 377).

Very little is known about the role that Clb-Cdc28 activity plays at origins. Upon activation of Clb5/6-Cdc28 and the DDK, a protein known as Cdc45 is loaded onto origins. Indeed, this recruitment appears to require CDK activity, however the nature of this dependence in not known (9, 10, 266, 346, 399, 400). A number of different studies have shown that Cdc45 plays an essential role in regulating DNA replication. It has been suggested that this protein may be involved in the loading of DNA polymerases onto origins (9, 233, 399). Cdc45 has also been shown to interact with the MCM complex, as well as Replication Protein A (RPA), suggesting that it may also be involved in coordinating the progression of replication forks throughout S-phase (163, 181, 308, 357, 399). Additionally, Cdc45 is also known to associate with a replication protein known as Sld3, presumably forming a complex at origins (163). Along with CDK activity, another protein complex that appears to regulate the association of Cdc45 to origins is the GINS complex (344). The GINS complex consists of Sld5, Psf1, Psf2 and Psf3, and has also been shown to associate with Sld3. Another protein shown to be essential for DNA replication is the Sld2 protein, which has been shown to interact with Dbp11 (a large subunit of DNA polymerase ε) (162, 344, 371). Interestingly, Sld2 proves to be a DNA replication-promoting target of Clb5-Cdc28 (220). Phosphorylation by Clb5-Cdc28 is required for the interaction between Sld2 and Dbp11, leading to the recruitment of DNA polymerase ε to origins (162, 220, 221). Sld2, however, is most definitely not the only essential Clb-Cdc28 target during replication initiation. Phosphomimetic (S/T-D) mutations in potential CDK consensus sites within Sld2 has been shown to allow Sld2 to function independently of Clb-Ccd28 activity, however these mutations do not bypass the requirement for Sic1 destruction at START (220). Initiation of replication has also been shown to require the displacement of Cdt1 and the destruction of Cdc6. The destruction of Cdc6 is believed to be mediated by Clb-Cdc28 activity, representing another potential role for Clb5-Cdc28 in origin firing (38, 76, 82). Indeed, Clb5-Cdc28 is able to specifically phosphorylate Cdc6 *in vitro* (202).

Once the Initiation Complex is fully assembled, DNA replication ensues. The first step is the activation of DNA polymerase α/primase which synthesizes an RNA primer at the origin on the leading strands (144, 145, 244). DNA polymerase ε and δ then commence DNA synthesis in a bi-directional manner from the origin on both the leading and lagging strands (319, 369).

DNA must only be replicated once per cell cycle, and hence, origins must only be fired once per cell cycle. Clb-Cdc28 activity plays a key role in regulating DNA synthesis, not only at initiation of replication, but also in its fidelity by specifically preventing re-replication during S, G_2 and M phases (17, 253). Essentially, CDK activity prevents reinitiation of replication by inhibiting the formation of pre-RCs. As mentioned, Clb-Cdc28 phosphorylates Cdc6, promoting its degradation in yeast (38, 76, 82). Also, phosphorylation-dependent binding of Clb2-Cdc28 to the N-terminus of Cdc6 may be another mechanism working to prevent rereplication in later stages of mitosis (232). Therefore, Clb-Cdc28 activity prevents reassembly of Cdc6 onto origins, ensuring that pre-RCs don't reform (38, 64, 75, 132, 277, 279). CDK activity also inhibits MCM function at origins, however it is not well understood exactly how. In budding yeast, inactivation of CDK activity causes the stable maintenance of Mcms within the nucleus, suggesting that Clb-Cdc28 may promote the nuclear export of Mcm proteins as a means of preventing the reformation of pre-RCs (129, 184, 252). In support of this, it has been demonstrated that CDKs are able to phosphorylate Mcm2 and Mcm4 (90, 97, 128, 154, 276). Finally, the ORC has also been shown to be targeted by CDK activity (253, 361). Mutations in the CDK consensus sites of Orc proteins can promote rereplication in yeast, suggesting that CDKs also modify ORC to prevent reassembly of pre-RCs. Clb5 has been shown to bind to Orc6 at origins of replication after origin firing, which is important to prevent rereplication (378). Interestingly, this interaction between Clb5 and Orc6 is not important for the initiation of DNA replication. Work in fission yeast has uncovered a similar mechanism to inhibit rereplication, which involves the binding of the B-type cyclin-CDK complex Cdc13-Cdc2 to origins in an ORC-dependent manner (382).

The G_2/M Transition: Following DNA replication, cells enter a second gap phase known as G_2. Before cells can continue through G_2 and enter M-phase, they must ensure that DNA replication has concluded successfully and that the DNA is undamaged. Fidelity of DNA replication is monitored throughout this process by regulatory networks known as checkpoints (81, 122). Checkpoints make sure that critical events in cell division are successfully carried out, and specifically work to maintain genetic stability. Checkpoints recruit a number of proteins which are involved in signaling pathways that are tied into cell cycle control, thus providing a means to halt progression of cell division until defects can be repaired. A well studied example of checkpoint action is the DNA damage checkpoint in yeast (81, 274). This checkpoint responds to various forms of DNA lesions (double strand and single strand breaks, nucleotide base modification or cross-linking etc…), and is functional in at least three distinct stages within the cell cycle, at the G_1/S boundary (G_1-phase checkpoint), within S-phase (intra-S-phase checkpoint), and at the G_2/M transition (G_2-phase checkpoint). In *S. cerevisiae*, a number of different proteins are involved in the DNA damage checkpoint including the Rad24 group of proteins (Rad24, Rad17, Mec3 and Ddc1), Rad9, Mec1 (ortholog of mammalian ATM/ATR kinases), Chk1, and Rad53 (ortholog of mammalian Chk2 kinase) (81, 375, 376). In metazoans, the G_2-phase DNA damage checkpoint has been shown to affect the regulation of cyclin B-Cdc2, preventing its activation, thereby inhibiting the entry into M-phase (1). A similar mechanism is proposed for the checkpoint response in *S. pombe* (394). Interestingly, budding yeast begin spindle assembly during S-phase, undergoing some of the early functions of M-phase during DNA replication (81). Maintenance of these assembled spindles requires an elevated level of Clb-Cdc28 activity, forcing budding yeast to develop a different strategy to halt cell cycle progression during a G_2-phase checkpoint response. Essentially, this checkpoint targets anaphase, preventing chromosomal division until defects have been repaired. It has been proposed that central regulators of anaphase, such as the Anaphase Promoting Complex (APC) and the securin Pds1, may be targets of this G_2/M checkpoint response (48, 81) (APC and its role in anaphase is discussed below).

After successful completion of DNA replication, a second wave of gene expression ensues to promote the G_2/M transition. As with late-G_1 gene expression, this G_2/M transcription is characterized by the expression of key cyclins which are involved in regulating M-phase. These cyclins are *CLB1, CLB2, CLB3* and *CLB4* (12, 380). *CLB3* and *CLB4* expression actually begins during the end of S-phase, peaking at G_2 and persisting until anaphase (83, 98, 245, 299). High-copy expression of these two cyclins has been shown to suppress the G_2-arresting phenotype of the *cdc28-1N* allele, implicating these cyclins as being involved in the G_2/M phase transition (342). Though it is still not understood exactly how Clbs promote this progression or regulate mitosis, some generalizations have been made. Clb3 and Clb4 seem to be involved in early mitotic events such as formation of mitotic spindles (91, 299). There is actually evidence to suggest that Clb5 (and maybe Clb6) may share some overlapping functions with Clb3 and Clb4 in this area. Even though *clb3, clb4* and *clb3 clb4* mutants display no apparent defective phenotype, *clb3 clb4 clb5* triple mutants cannot assemble spindles and are inviable (91, 299, 316). However, these triple mutants might actually display compounded defects in DNA replication and spindle assembly, since Clb3 and Clb4 are known to function in S-phase initiation in cells lacking *CLB5* and *CLB6* (299). *CLB1* and *CLB2* are the next and final pair of B-type cyclins to be expressed, displaying a distinct periodicity during mitosis by strongly peaking just before anaphase (91, 299). *CLB1* and *CLB2* were also shown to be high-copy suppressors of the *cdc28-1N* phenotype (342), and have been suggested to function in the regulation of nuclear division (299). Studies on mutants have suggested that Clb2 may be the principle B-type cyclin in *S. cerevisiae*. Unlike the other *CLB*s, *clb2* single mutants display a fairly strong phenotype, resulting in larger cells with a greater proportion of the population being G_2 budded cells (91, 299). Also, *clb1 clb2* or *clb2 clb3* double mutants are inviable. In contrast, *clb1* mutants show no phenotype, and in fact, *clb1 clb3 clb4* triple mutants display only mild mitotic defects.

Regulation of the gene expression seen during the G_2/M transition has recently become fairly well characterized. The genes that fall under this wave of expression have been classified more specifically as the “Clb2 cluster” (106, 342).

Besides *CLB1* and *CLB2*, some other genes represented in this Clb2 cluster include the transcription factors *SWI5* and *ACE2*, and the polo-like kinase *CDC5*. Studies on the *SWI5, CLB1* and *CLB2* promoters had demonstrated early on that the MADs-box transcription factor Mcm1 was required for their periodic expression (6, 206, 209). It was also discovered that when Mcm1 was bound to the *SWI5* promoter, it would associate with a protein complex initially designated as the *SWI5* factor (SFF). Recent work has demonstrated that SFF binding sites are recognized by the forkhead family transcription factors (138, 182, 283). Two members in particular are required for the regulation of G_2/M gene expression, Fkh1 and Fkh2 (137, 177, 182, 398). Fkh2 proves to be the main factor forming a complex with Mcm1 at Clb2 cluster gene promoters (137, 138), and this complex remains bound constitutively to their promoters throughout the cell cycle (6, 177). The periodic expression of Mcm1-Fkh2 regulated genes has been shown to be dependent on Ndd1, which acts as an activator in this transcription factor complex (177, 204, 296). Ndd1 is expressed periodically beginning at S-phase and persists until its turnover during M-phase (204). An intricate mechanism has recently been proposed detailing the regulation of the Mcm1-Fkh2-Ndd1 transcription factor complex (282). Clb5-Cdc28 may phosphorylate promoter bound Fkh2, resulting in the recruitment of Ndd1 to the Mcm1-Fkh2 complex, thereby leading to the activation of this transcription factor. Further stabilization of this complex through phosphorylation of Ndd1 by the induced expression of Clb2 may serve as a positive feedback mechanism enhancing the G_2/M gene expression. It should be noted that the regulation of *CLB3* and *CLB4* expression still remains uncharacterized. However, Fkh1 has been shown to bind to the *CLB4* promoter, suggesting a role for this transcription factor in early-G_2 gene expression (324).

Description of the G_2/M transition presented in this section has illuminated a cascade of regulation beginning with key products of G_1/S transcription, which function to promote the progression towards M-phase. Figure I-2 diagrams the regulatory network seen during the G_2/M transition and outlines the key regulators involved.

Even though their exact activities remain unclear, Clb1-4 appear to play important roles in mitotic spindle regulation, and thus are factors controlling chromosomal segregation in M-phase. Recent work has shown that Cdc28 activity is important in regulating the DASH complex (Dad1, Dad3, Dam1, Duo1, Ask1, Spc19, Spc34, and Hsk1/Dad2), which mediates the interaction between the spindle and the kinetochore (194). It is believed that Clb-Cdc28 phosphorylates Ask1, which is a component of the DASH complex, promoting its full activity. Regulation of the mitotic spindle is an integral part of the dynamics of this process. The spindle is composed of microtubules, which are polar dynamic fibers polymerized from tubulin subunits and other important components (99, 330). These microtubules are arranged in a bipolar array, uniformly oriented with their minus ends originating at the spindle poles, and their plus ends extending away. Microtubules from opposite spindle poles attach to duplicated chromosomes via a pair of specialized structures at the centromere known as kinetochores. Bipolar kinetochore-microtubule attachments are favored, producing the most stable interactions. This permits the directed movement of chromosomes along the spindle, leading to alignment at the metaphase plate (123). Bioriented chromosomes fall under tension which acts as a biochemical signal indicating proper spindle-chromosome attachments. This tension, which is produced by spindle-forces acting in opposite directions on chromosomes, is dependent on intricate connections that exist between sister-chromatids ("chromatid cohesion") (247). Chromatid cohesion proves to be important for maintaining genetic integrity, for allowing proper chromosomal alignments, and is the focal point for the initiation of chromosome segregation and mitotic exit. Therefore, a closer look at chromatid cohesion is warranted.

Sister-chromatid cohesion originates upon DNA replication, and persists until chromosome alignment at metaphase (247). In *S. cerevisiae*, cohesion relies on the association of four proteins (Smc1, Smc3, Scc1 and Scc3) which form a complex termed "cohesin" (111, 203, 230, 351). Scc1, Smc1 and Smc3 are found tightly bound to specific sites along chromosomes, usually corresponding to inter-genic regions (89, 108, 191). It has been shown that Smc1 and Smc3 form a V-shaped heterodimer (226). Essentially, both Smc1 and Smc3 fold back onto themselves producing a coiled-coil region, with a globular ATP-binding head at one end and a heterodimerization domain at the other (113, 135). Smc1 and Smc3 associate with each other through these heterodimerization domains to form the V-shaped structure. Scc1 is believed to connect the globular heads of this V-shaped structure together, producing a closed triangular ring, and Scc3 is thought to be recruited to this ring through association with Scc1. The exact mechanism by which the cohesin complex contacts the sister-chromatids or maintains cohesion is not well understood. Many models have been proposed, including those involving physical interactions between the globular head domain and the sister-chromatids, or topological interactions involving the entrapment of chromatids within a single cohesin ring (247). However, the importance of the cohesin complex in maintaining sister-chromatid cohesion, as well as in the regulation of chromosome segregation remains clear.

The M/G$_1$ Transition: It has long been known that cyclin degradation is a requirement for the completion of cell division (86, 106, 205, 239, 240). It has also long been known that cyclin-CDK activity regulates cyclin destruction, limiting it to the exit of mitosis. Cyclin destruction has been shown to be dependent on the presence of sequence motifs found in cyclin proteins known as "destruction boxes" and "KEN boxes" (88, 107, 240, 281). Based on the destruction box motif defined in cyclin B and cyclin A, similar sequences have been found near the N-terminus of Clb1, Clb2, Clb3, Clb4 and Clb5 (83, 91, 180, 316). The KEN box has also been shown to be involved in Clb proteolysis during mitotic exit (127). The destruction box and the KEN box are recognized by a multi-protein ubiquitin ligase (E3) known as the cyclosome or Anaphase Promoting Complex (APC). The APC was originally defined in *Xenopus* and clam oocytes as the factor responsible for ubiquitination of cyclin A and cyclin B, targeting these cyclins for destruction. The APC is also responsible for the destruction of Clbs during anaphase in *S. cerevisiae* (116). Mutations in APC subunits produce significant phenotypes in yeast, arresting as large-budded cells with replicated but undivided nuclei (125, 152, 178, 322, 391-393). This phenotype is consistent with a defect in chromosome segregation, and the inability to destroy Clb cyclins. Just as the SCF requires various F-box proteins to mediate target specificity, the APC also requires variable targeting factors to modify different substrates. Two of these factors are the related proteins Cdh1 and Cdc20 (314, 317, 368). However, unlike the SCF which requires prior phosphorylation of its targets, the APC relies on regulation of Cdc20 and Cdh1 for its activity towards intended substrates (368). Cdc20 is required for APC activity during mitosis, while Cdh1 is needed during late mitosis and in G_1. A very simplistic view of APC regulation as it pertains to Clb regulation is described here (116). During M/G_1, APC^{Cdh1} activity ensures Clb instability, which is a pre-requisite for the formation of pre-RCs at replication origins (see S-phase). Upon START, activated Cln-Cdc28 and Clb5/6-Cdc28 kinases phosphorylate Cdh1, promoting its disassociation from the APC leaving this E3 inactive. This allows a rise in Clb cyclin abundance. During M-phase, as Clb2-Cdc28 levels rise, this kinase proceeds to phosphorylate Cdc20, thereby promoting its association with the APC. This activated APC^{Cdc20} promotes the proteolysis of Clb5 (and Clb3), relieving the inhibition on Cdh1. Therefore, coincident with the onset of anaphase, APC^{Cdc20} and APC^{Cdh1} work together to degrade the Clbs and promote mitotic exit. This process also requires the activity of the Cdc14 phosphatase and

Sic1. Cdc14 maintains Cdh1 and Sic1 in their dephosphorylated states, thereby keeping them active towards inhibition of Clb-Cdc28 activity. Another factor promoting the inactivation of Clb-Cdc28 during mitotic exit is the protein kinase Swe1 (*Saccharomyces* Wee1) (23). Swe1 phosphorylates Cdc28 on a specific tyrosine residue (Y19) and inhibits its activity in late M-phase

Along with Clb destruction during mitosis, the APC^{Cdc20} plays another essential role in promoting mitotic exit – the segregation of sister chromatids. Once activated, APC^{Cdc20} marks the *S. cerevisiae* Pds1 (securin) for destruction (50, 247, 386, 387). Pds1 acts as an inhibitor chaperone to the separase Esp1 (a cysteine protease). Free from Pds1 inhibition, Esp1 cleaves the cohesion complex subunit Scc1, eliminating cohesion between sister chromatids and thereby promoting chromosome segregation (358, 359).

In essence, the initiation of anaphase promotes the mitotic exit program, characterized by the afore-mentioned destruction of Clb cyclins and the division of chromosomes into mother and daughter cell bodies, followed by cytokinesis and budding. During mitotic exit, one more wave of transcription has been identified, essentially serving to reset conditions necessary for the onset of a new cell division cycle. The genes involved in this wave of expression are termed M/G_1 genes, and are also dependent on the MADs-box transcription factor Mcm1 (12). Many genes in the M/G_1 class have been show to contain a promoter element called the early-cell-cycle box (ECB), which serves as a Mcm1-binding site (225, 334). Examples of M/G_1 genes regulated by Mcm1 are the SBF subunit *SWI4*, the G_1-cyclin *CLN3*, and the DNA replication protein *CDC6*. Mcm1 binds constitutively to the ECB site (208, 210), and relies on transcriptional repression of its targets to limit its activity to M/G_1 (288). The homeodomain proteins Yox1 and Yhp1 have been reported to bind to typical homeodomain binding sites found upstream of Mcm1 sites within the ECB, and act as repressors of Mcm1-mediated M/G_1 genes. Other transcription factors regulating M/G_1 genes are the related proteins Swi5 and Ace2 (expressed in an Mcm1-dependent manner at G_2/M) (71). Swi5 and Ace2 targets include genes required for cytokinesis, as well as genes needed during G_1 such as Sic1 (173). Figure I-3 diagrams the regulation of the M/G_1 transition described here.

A Last Look at Mitosis: As is strikingly apparent from the discussion of the *Saccharomyces cerevisiae* cell cycle above, transcriptional regulation proves to be the key to promoting processes, not only within a given stage, but transitions from one stage to the next. Figure I-4 presents a basic schematic diagramming the progression through the cell cycle, and outlining the key transcription factors involved. Indeed, a transcriptional cascade emerges as the underlying mechanism, whereby transcription factors acting in one phase of the cell cycle lead to expression of transcription factors required to promote events in the next phase.

I.2 – Alternate Cell Fates

The absolute foundation for a living cell is the basic cell cycle, which is required for growth and proliferation. However, for the diversification and adaptation of living organisms, cellular processes must become specialized and succumb to differentially regulated states as a response to the cell's surroundings and certain genetic cues. This leads to increased survivability and complexity in the living organism as a whole. Two underlying principles can be considered as key mechanisms promoting cellular development and specialization: the malleability of the cell cycle, and the variability within the architecture of gene regulatory networks. As already discussed, the ultimate progression of the cell cycle is subject to stringent controls ensuring fidelity of this intricate process. However, regulation of the mechanisms driving the cell cycle appears to be somewhat flexible. From altering the duration of specific stages within cell division, to altering features of a stage altogether, variable regulation of the cell cycle is an absolute requirement for cellular specialization. The course of a eukaryotic organism's development relies on large gene regulatory networks. These gene networks can be defined as functional linkages among regulatory genes whose products regulate other targets producing a hierarchical cascade of gene expression (61). This cascade works towards a terminal purpose, resulting in morphological and functional consequences. And so, together, the flexibility of the cell cycle and the diversity of gene expression are employed towards the emergence of a number of different cell fates seen in eukaryotes. A few examples of these cell fates are discussed below.

I.2.a. - Metazoan Development

The development of multicellular eukaryotes offers a prime example of cellular specialization. Development involves variable regulation of cellular processes to produce the final somatic body plan. A well documented model organism for developmental research is the small roundworm *Caenorhabditis elegans* (170). Upon fertilization of the oocyte, the initial cell cycles during embryogenesis lack G_1 and G_2 phases, only involving consecutive rounds of DNA replication (S-phase) and mitosis (M-phase) (79). During this rapid cleavage-type division, cell volume of the large zygote decreases with every division, a feature common among many metazoan species. These early embryonic cell cycles actually lack stringent check point controls (169, 170, 294). In *C. elegans*, a G_2-phase first emerges in the 24-cell stage embryo in a specific cell lineage (79). In fact, it is theorized that the acquisition of the G_2-phase may also coincide with the introduction of checkpoint controls. Eventually, in the late-dividing embryonic cells, a clear G_1-phase is seen indicating the incorporation of G_1-phase regulators such as G_1-cyclin-CDKs (cylin D-CDK4) and their inhibitors (*cki-1* and *cki-2*) (26, 271). Every cell in the developing *C. elegans* has a defined role, representing a distinct cell lineage (170). Interestingly, during larval developmental stages, two tissues (the intestines and hypodermis) undergo endoreduplication, whereby mitosis is bypassed producing a G_1-phase nucleus with double the genomic DNA

(124). In fact, these tissues undergo multiple rounds of endoreduplication producing 32n nuclei. This process allows for the specialization of these tissues to maintain integrity of the organs they develop into while allowing for the increased genome ploidy needed to support a greater cell volume and metabolic activity.

I.2.b. - Quiescence/G_0

Both prokaryotic and eukaryotic cells demonstrate the ability to leave normal cell cycle progression and enter a resting state known as quiescence or G_0 (110). This state serves a number of different purposes, from survival in microorganisms to terminal differentiation in many metazoan tissues. Switching between active proliferation and quiescence is believed to involve the complete reprogramming of gene regulatory networks and the remodeling of many intracellular processes such as the cell cycle machinery. The yeast *Saccharomyces cerevisiae* serves as an established model organism in quiescence/G_0 research due to the conservation of many basic cellular processes and regulatory machinery among eukaryotes. Entry into G_0 in yeast occurs before the START commitment step, in the presence of certain starvation conditions (119, 260). Only upon favorable changes in nutrient availability do cells exit G_0 and reenter G_1 of the cell cycle. Many signaling pathways converge to control entry into quiescence in yeast. The TOR and protein kinase A (PKA) pathways serve as negative regulators of quiescence, while the protein kinase C (PKC) and Snf1 pathways serve as positive regulators of quiescence (110). These signaling pathways coordinate to regulate the expression of genes required for the "cell quiescence cycle" (ie. entry into and exit from G_0).

Even though it is not well characterized, the progression of cells through proliferation is prevented during G_0. One possible method to inhibit cell cycle progression could be to block the G_1/S transition. TAFII145, the core subunit of the TAFII complex (an associated factor of the general transcription factor TFIID) is dispensable for most gene expression in yeast, but is absolutely required for the G_1/S transition (370). It has been reported that levels of TAFII145, other TAFIIs, and the TATA box-binding protein are dramatically reduced in quiescent cells. Another target of G_0 regulation, that could work to block the G_1/S transition, is the ribosome (110). Protein synthesis requires a large reserve of energy, and the expression of genes encoding translational machinery represents about 75% of the total transcription during the cell cycle. Therefore, upon the transition into G_0, a global shutdown of ribosome genes occurs. And since the rate of protein synthesis is directly correlated to the regulation of START, negatively regulating ribosome function would inhibit this transition.

Cells which are stimulated to exit quiescence by refeeding undergo a rapid transcriptional response (110). The greatest changes in expression occur within the first 10-15 minutes post-induction, and throughout the process the expression of about one-third of all genes is affected. Distinct temporal stages of expression are observed implying that exit from G_0 involves a sequential series of events.

Even though some genes characteristically expressed during G_1 are rapidly induced upon the addition of nutrients (eg *SWI4*), cells exiting from G_0 appear to proceed through unique states rather than immediately entering G_1.

To date, very little is really known about quiescence in any organism. The mechanisms controlling entry, maintenance, and exit from G_0 are poorly understood. But evidence does support a unique quiescence program, involving specific gene expression and intracellular events leading to a distinct cell fate.

I.2.c. – Meiotic Development

The chromosome theory of Mendelian heredity is based on the fundamental principle that discreet genetic determinates (ie chromosomes), one maternal and one paternal, are acquired upon conception in sexually reproducing organisms (39). To maintain ploidy of the organism, haploid gametes are generated from diploid somatic cells through a specialized cell division cycle termed meiotic development. Gametes, which in animals constitute sperm (paternal) and ova (maternal), maintain species continuity by conveying parental genes to the next generation of progeny. This is accomplished by fusion of these haploid gametes to produce a diploid zygote, which serves to regenerate the diploid chromosome number, and facilitate reproduction. Generally, the reduction of chromosome number during gametogenesis is accomplished by one round of DNA replication, pairing of homologous chromosomes and recombination, followed by two consecutive rounds of chromosomal divisions.

During the first meiotic division, called Meiosis I (MI), homologous pairs are segregated (reductional division). During the second meiotic division, called Meiosis II (MII), sister chromatids are segregated (equational division). Meiosis relies on much of the same general machinery involved in driving the mitotic cell cycle, however unique meiosis-specific genes and regulatory networks are activated to perform this specialized process. Also, significant reorganization of the cell cycle is required to achieve gamete formation. As meiosis is a central focus for studies encompassing the graduate work presented in this thesis, an overview of meiosis in budding yeast is warranted.

Sporulation in Budding Yeast: Gametogenesis in diploid *Saccharomyces cerevisiae*, known as sporulation, involves the formation of four haploid spores housed in an ascus (called a tetrad) (234). Two mating types exist among yeast, termed type **a** and type α. Under the right conditions, haploid spores will germinate and fuse to a haploid of opposite mating type to reproduce an **a/α** diploid cell. The initiation of sporulation depends on specific genetic and environmental conditions (140). Understandably, only diploid cells may undergo meiotic development. Both **a** and α cell type specific factors form a complex, and are directly involved in promoting sporulation under the appropriate conditions. Signals arising from environmental cues mediating the initiation of meiosis in yeast are instigated by such conditions as nutrient starvation (eg nitrogen limitation), the presence of a non-fermentable carbon sources (eg acetate or ethanol), and glucose signals (which inhibit sporulation) (140). In multicellular organisms meiotic induction is initiated from extrinsic cues derived from surrounding cells. The molecular mechanisms that respond to these cues and regulate the meiotic developmental process are not as well established as those described in yeast (214).

Sporulation in budding yeast is generally characterized by a cascade of gene expression, subdivided into at least four sets of genes: early, middle, mid-late, and late genes (46, 234). Meiotic induction initiates the expression of early genes, which are mostly involved in early events such as premeiotic DNA replication, homologous chromosome pairing and meiotic recombination (303). The products of middle genes regulate the processes of meiotic nuclear divisions and the initiation of spore formation (47, 96). The mid-late and late genes are required for the formation of the outer spore wall and final spore maturation events (33, 186). As in the mitotic cell cycle, the coordinated expression of key regulatory factors in meiosis drives the events within a specific stage, as well as the transition from one stage to the next.

Meiotic Induction and Early Gene Expression: Upon induction of meiotic development, the nutritional signaling pathways promoting sporulation converge on the transcriptional regulation of two key regulators of meiotic initiation: *IME1* (Initiator of Meiosis 1), which encodes a transcription factor, and *IME2*, which encodes a Ser/Thr protein kinase (140, 365). The activity of Ime1, also called the "master regulatory switch" for meiotic initiation, is intricately regulated at both the transcriptional and post-transcriptional levels (164, 327). Genetic and nutritional signals directly target regulatory elements in the unusually large *IME1* promoter to regulate its expression (55, 306, 307). In haploids, *IME1* is repressed by a complex which comprises the zinc-finger repressor protein Rme1, and components of the RNA polymerase mediator complex, Sin4 and Rgr1 (54-56). In diploid cells, a complex composed of the cell type specific proteins a1 and α2 represses the expression of *RME1*, thereby relieving the Rme1-mediated repression on the *IME1* promoter (54, 338). It has also been shown that Rme1 acts as a positive regulator of G_1-cyclin expression in haploids (350). G_1-cyclins inhibit the induction of meiosis, by inhibiting the expression of *IME1* (52, 292). Therefore, repressing *RME1* further enhances the induction of sporulation by down-regulating *CLN* transcription. The a1/α2 complex also promotes the expression of *IME4*, whose gene product is a positive regulator of *IME1* expression (318). Far less is understood about the nutritional signals feeding into regulation of *IME1* transcription. Mutational analysis of the *IME1* promoter has identified a number of regulatory elements responding to nutritional cues, such as glucose starvation and the presence of acetate (140, 307, 365). In low levels of glucose, for instance, cells activate the RAS-cAMP pathway which promotes the binding of the transcription factors Msn2 and Msn4 to stress response elements (STRE) in the *IME1* promoter (223, 307).

The activation of Ime1 kick-starts a cascade of meiotic gene expression promoting progression through sporulation (234). As already mentioned, Ime1 is a transcription factor which has been shown to directly regulate many early meiotic genes (140, 365). Early gene expression begins 0.5 to 2 hours after meiotic induction, and a large number of these early genes maintain a conserved element in their promoters called the URS1 (Upstream Repressor Sequence) (35, 234). Early meiotic genes are repressed during vegetative growth by a repressor complex that binds to the URS1 (159, 160, 305, 372). This repressor complex is composed of the zinc-finger DNA-binding protein Ume6, the co-repressor Sin3, and the histone-deacetylase Rpd3. Upon induction of sporulation, the Sin3-Rpd3 complex is inactivated by an unknown mechanism and possibly disassociates from Ume6. The URS1 bound Ume6 is then converted to a transcriptional activator through an association with Ime1.

Post-transcriptional modification of Ime1 and Ume6 by the Gsk3 family kinase Rim11, and possibly the Rim15 kinase, stabilizes the Ime1-Ume6 interaction, fully activating its transcriptional activity (211, 212, 304, 366, 383). It is important to note that nitrogen limitation and glucose starvation activates Rim11 and Rim15, further enhancing the nutrient response signals promoting Ime1 activity. Microarray analysis has identified about 62 genes demonstrating early expression in sporulation (46). Almost half of these early genes maintain a URS1 core consensus sequence in their promoters and are thought to be regulated by Ime1. One of the most critical Ime1 targets is the meiosis-specific Ser/Thr protein kinase encoded by *IME2* (235). Figure I-5 depicts the regulatory network that initiates the meiotic induction program, focusing on the activation of Ime1 and the expression of *IME2*.

Premeiotic S-phase: Ime2 activity is absolutely essential at various stages throughout meiotic progression (92, 176, 235, 388). One of its first critical roles is in the control of the meiotic G_1/S transition (19, 70, 92, 112). *ime2* mutants arrest in G_1 of meiosis and fail to initiate DNA replication. As in mitosis, Clb5/6-Cdc28 activity regulates premeiotic DNA replication and is inhibited by Sic1 (19, 70, 340). Hyperstable Sic1ΔP has been shown to block premeiotic DNA replication, demonstrating the need for Sic1 destruction in the initiation of meiotic S-phase (340). This is consistent with the regulation of Sic1 during mitotic DNA replication. However, unlike mitosis, G_1-cyclins are not expressed during sporulation and *cln1 cln2 cln3* mutant diploids perform meiosis successfully, producing viable spores (52, 70, 100, 114, 143, 273, 340). Therefore, to promote premeiotic S-phase, Sic1 degradation is thought to be mediated by an Ime2-dependent mechanism (70). To date, direct modification of Sic1 by Ime2 has not been demonstrated, however Sic1 remains stable in *ime2* mutants, and deletion of *SIC1* suppresses the *ime2* DNA replication defect (70). This has led to the proposal that Ime2 fulfils the role of G_1 cyclin-CDKs during meiotic development in budding yeast. Interestingly, Ime2 shares some sequence similarity to CDKs (146). Specifically, Ime2 and CDKs contain conserved amino acid residues involved in nucleotide binding and formation of a catalytic triad needed for the phosphorylation of target proteins. Ime2 also shares conserved residues within a putative activation loop (T-loop) found in CDKs and MAP kinases (102, 148, 331). In fact, evidence exists demonstrating that Ime2 activation requires the CDK-activating kinase Cak1 (312). The constitutively active Cak1 phosphorylates Cdc28 in the activation T-loop domain, and this modification is essential for CDK activity (85, 161, 348). It has been proposed that a similar modification by Cak1 in the putative T-loop of Ime2 may occur, serving to activate this meiosis-specific kinase upon its expression (312). Though these similarities are apparent, some striking contrasts distinguish Ime2 from the CDKs. Ime2 lacks a PSTAIRE motif which is conserved among CDKs and is required for cyclin binding (27). This would suggest that Ime2 does not require an activating/targeting subunit for its function. Ime2 has the ability to autophosphorylate, suggesting a positive feedback mechanism may contribute to its activation (19, 332). Also, Ime2 is highly unstable, containing an extended C-terminal domain possibly involved in mediated degradation (74, 112, 176).

Inactivation of the Clb-Cdc28 inhibitor Sic1 may not be the only role Ime2 plays in promoting DNA replication. During premeiotic S-phase, a meiosis-specific co-activator of the APC called Ama1 is expressed. To ensure the stabilization of Clb cyclins during meiotic S-phase and prophase I, APC^{Ama1} activity must be inhibited at these times. It has been suggested that Ime2 may function to inhibit the APC during meiotic development (22). However, this proposal is based on the analysis of ectopic Ime2 expression in G_1 arrested mitotic cells, where *AMA1* is not expressed, and instead focuses on potential activity against Cdh1. The role of Cdh1 during sporulation is not well characterized. Recently, it has been shown that a subunit of the APC, Mnd2, actually functions to inhibit APC^{Ama1} activity during meiotic development, a role it does not play during mitotic proliferation (263). Whether or not Ime2 functions to regulate Mnd2 activity has not been addressed.

As mentioned, meiotic S-phase is also regulated by Clb5/6-Cdc28 activity (19, 70, 340). However, in striking contrast to mitotic S-phase, Clb5 and Clb6 are absolutely essential for promoting premeiotic DNA replication (340). Sporulating *clb5 clb6* mutants fail to activate meiotic DNA synthesis even though *CLB1-4* expression during sporulation is not significantly affected. This suggests that the functional redundancy demonstrated by Clb1-4 in *clb5 clb6* cells during the mitotic cell cycle does not seem to be employed during sporulation. The reason this differential requirement for Clb5 and Clb6 is seen in meiotic S-phase remains unclear. Specificity in cyclin substrate recognition or specialization in cyclin targeting may provide an explanation. Variation in the dynamics of S-phase in meiosis versus mitosis may also play a role in the specific need for Clb5 and Clb6 (for further discussion and analysis of the essential requirement for Clb5 and Clb6 in premeiotic DNA replication see Chapter IV).

Apparent differences are observed when premeiotic S-phase is compared to its equivalent in mitotic proliferation. For instance, the initiation of DNA replication does not occur as rapidly upon meiotic induction as it does during mitotic cell cycle (40). Along with the differences in gene expression already discussed, evidence exists to suggest that pre-RC formation and mechanisms promoting initiation and elongation in premeiotic S-phase may differ from mitosis (93). Even though DNA replication seems to initiate from the same origins in meiosis and growth (51), the way in which origin firing is regulated might vary. Nonetheless, many of the core components that comprise the pre-RC in proliferation are also required during sporulation, such as the origin recognition complex (238), MCM complex (196, 238), Cdc6 (264), and the Dbf4-Cdc7 kinase (360). But, to date, very little work has been done outlining the regulation of pre-RC formation and origin firing during premeiotic DNA replication. Experiments investigating the activity of Dbf4-Cdc7 during sporulation have demonstrated a dual role for this kinase (360). Along with an essential requirement for promoting premeiotic S-phase (which is presumed to function in a similar manner to mitotic S-phase), Dbf4-Cdc7 also appears to be involved in aspects of the reductional MI division. Evidence also suggests that Cdc6 may be regulated differently in meiosis versus mitosis (264). As in mitotic S-phase initiation, Cdc6 is specifically degraded upon entry into meiotic S-phase, however origin bound Cdc6 seems to persist throughout sporulation and is protected from Cdc28-mediated inactivation. Interestingly, unlike in mitotic growth, where Clb-Cdc28 activity inhibits reformation of the pre-RC, overexpression of Clb1-Cdc28, or improper regulation of Cdc28 activity (via expression of the dominant *CDC28AF* allele) promotes multiple rounds of rereplication (297, 339). Though it has not been addressed, the persistence of Cdc6 at activated origins during sporulation may explain the rereplication phenotype seen in these studies.

Another feature unique to premeiotic DNA synthesis is the increased duration of meiotic S-phase (40, 51). This universal property may be due to mechanisms involved in pairing of homologous chromosomes and preparations for meiotic recombination (40, 373).

Meiotic Prophase I and Homologous Recombination: Prophase I of meiosis has been characterized by the global changes seen in chromosomes after premeiotic DNA replication, and has been divided into four separate stages: leptotene, zygotene, pachytene and diplotene (4). One of the hallmarks of meiotic development is the high frequency of homologous recombination seen during this lengthy prophase I (14, 179). Homologous recombination during meiotic development serves two important roles. First, it establishes physical connections between homologous chromosomes ensuring their correct alignment and disjunction during the first meiotic division (MI). And second, the increased level of recombination seen during meiosis also contributes to the genetic diversity required to sustain a healthy population. In yeast, the regulation of meiotic recombination is linked to premeiotic DNA replication (36, 311, 323, 340). Homologous recombination depends on the successful completion of premeiotic DNA replication, and disruption of DNA synthesis prevents recombination (24, 40, 62, 329, 340). Though this dependence may reflect a checkpoint, many lines of evidence seem to indicate that this is not the case. Rather, a mechanistic connection between premeiotic DNA replication and meiotic recombination appears to be more a reasonable scenario. For example, the budding yeast gene *MUM2*, shown to be essential for premeiotic DNA replication but not required during mitosis, is also needed for meiotic recombination (62). Another feature facilitating the dependence of homologue pairing and recombination on the passage through meiotic S-phase involves chromosome cohesion via the cohesin complex (172, 237). It has been suggested that cohesin must be laid down during premeiotic S-phase to support the interactions between homologues (373). The cohesin complex, and its role in meiotic chromosome segregation will be discussed below (see "From Prophase I to Middle Sporulation").

Homologous recombination is a complex process involving a number of dynamic chromosomal events. During leptotene, alignment of homologous chromosomes occurs, whereupon the formation of axial elements along chromosome arms and early recombination nodules begins (298). The initiation of meiotic recombination in budding yeast relies on the activity of the topoisomerase-like transesterase Spo11 (165, 166, 328). Spo11 forms a number of programmed double-strand breaks (DSBs) at numerous sites within the genome. This process also involves at least nine other proteins and two meiosis specific RNA-splicing factors (165). Repair of these DSBs by homologous recombination ensues, favoring interactions between non-sister chromatids. Essentially, the processing of DSBs leads to exposed single stranded tails with 3' ends, which then invade homologous sequences in non-sister chromatids (298). Strand invasion forms a Holiday Junction and recombination proceeds through zygotene and into early pachytene. Complete chromosome synapsis occurs during late pachytene through the formation of the synaptonemal complex. The transition from pachytene to diplotene is marked by the degradation of the synaptonemal complex, leaving behind visible chiasmata (potential sites of crossover).

From Prophase I to Middle Sporulation: A key feature ensuring the proper completion of recombination in yeast is the initiation of the pachytene checkpoint (also called the recombination checkpoint), which is activated by Spo11-mediated DSB formation (166). Just as the mitotic checkpoints functioning in G_2 prevent mitotic chromosomal segregation until DNA replication has successfully completed, the pachytene checkpoint functions in meiotic prophase I to inhibit meiotic nuclear divisions until cells have successfully completed recombination and chromosome synapsis. In fact, checkpoint proteins such as Mre11 have been shown to interact with the synaptonemal complex, and be directly involved in the resolution of DSBs (300). Defects in this important control mechanism can lead to problems such as missegregation of chromosomes resulting in the generation of aneuploid gametes.

A number of different proteins are involved in activating or coordinating the pachytene checkpoint. These proteins fall into different classes such as DNA-damage checkpoint proteins, meiotic chromosomal proteins, recombination proteins, chromatin silencing factors, and cell cycle proteins (300). Of the DNA-damage checkpoint proteins, the Rad24 group of proteins (Rad24, Rad17, Mec3, Ddc1 and Mec1) are known to play a role in the pachytene checkpoint (13, 207, 309). However, Rad9, Rad53 and Chk1 are not needed for this arrest (207). Rad24 and the Ddc1 complex (Ddc1, Rad17, Mec3) show homology to DNA binding proteins (241), and it is believed that they are sensors of DNA damage (397). Rad24 may recognize DNA lesions (such as DSBs) and recruit the Ddc1 complex, generating checkpoint signals promoting the pachytene arrest. Many yeast mutants of genes encoding chromosomal and recombination proteins, such as *zip1*, *hop2* and *dmc1*, have proven important for characterizing the pachytene checkpoint. Zip1 is a synaptonemal complex protein (343). *hop2* mutants fail to repair DSBs and many chromosomes in these cells are seen to synapse with non-homologous partners (192). Dmc1 is a meiosis-specific homolog of the *E. coli* protein RecA (20). In *dmc1* mutants, DSB repair is impaired, leading to hyperresected ends forming long single-stranded tails. The pachytene checkpoint is an essential control mechanism for meiotic development, which is evidenced by its conservation in higher eukaryotes, such as mice, *C. elegans* and *Drosophila* (300).

As already mentioned, the ultimate goal of the pachytene checkpoint is to arrest cells in pachytene, and therefore to halt the progression of the meiotic cell cycle. This would indicate that proteins central to the regulation of meiotic progression would also be involved in this checkpoint. In budding yeast, two downstream targets of the pachytene checkpoint promoting this arrest have been described: the CDK-inhibiting kinase Swe1, and the meiosis-specific transcription factor Ndt80 (300). As described in the discussion of mitosis above, the Swe1 kinase phosphorylates and inactivates Cdc28 during mitotic exit (23). In meiosis, when the pachytene checkpoint is activated, Swe1 accumulates and becomes hyperphosphorylated, ultimately leading to the inactivation of Cdc28 (193). Since Cdc28 activity is required for regulation of MI and MII, this inhibition ensures a pachytene arrest. The second target of the pachytene checkpoint, Ndt80, is a meiosis-specific transcription factor and a key regulator of meiosis (47, 131, 269, 352). *ndt80* mutants arrest at pachytene with fully synapsed chromosomes and demonstrate no defects in recombination (385). These mutants also show a dramatic reduction in the expression of both middle and late meiosis-specific genes (47, 131). To fully understand the significance of Ndt80 as a target of the pachytene checkpoint, an evaluation of its regulation is in order.

Ndt80 is one of the key components of the transcriptional cascade driving meiotic development, and is a central regulator of middle sporulation gene expression (47). About 158 genes are strongly induced during middle sporulation (between 2 – 5 hours post induction) (46). Almost 70% of these genes contain a putative regulatory element in their promoters known as a Middle Sporulation Element (MSE). The MSE was first identified as being necessary for meiosis-specific expression of its regulated genes during middle sporulation (130, 267), and has been shown to be a specific binding site for Ndt80 (47, 333). Key targets of Ndt80 transcriptional activity are the B-type cyclins *CLB1, CLB3, CLB4, CLB5* and *CLB6*, which are also required for meiotic nuclear divisions (47). Expression of *NDT80* actually begins early in sporulation, where low levels of this transcript are detected (268). This early expression is induced by the Ime1/Ume6 complex through URS1 elements in the *NDT80* promoter. *NDT80* also contains MSEs in its promoter, and is strongly expressed during middle sporulation, employing a positive feed-back mechanism to regulate its own transcription (47, 268). Regulation of middle-sporulation genes also involves the activity of transcriptional repressors, Sum1 and the Set3 complex, which inhibit the expression of these genes during vegetative growth and early meiosis (286, 384). In fact, Sum1 specifically represses Ndt80-regulated genes, including *NDT80* itself, by competing for an overlapping binding site in the promoters of these targets (284). In many cases, Sum1 has been shown to recruit the NAD+-dependent histone deacetylase Hst1 to support its repressive activity (224, 268, 384). It has been reported that levels of Sum1 protein decrease dramatically upon the onset of middle sporulation, possibly mediated by a post-transcriptional mechanism (195).

Another interesting aspect of Ndt80 regulation is that full transcriptional activity, which is required for the strong induction of itself and its targets, has been shown to require the meiosis-specific kinase Ime2 (268, 332). It has been reported that Ndt80 exists as a phosphoprotein *in vivo* (352). Ectopically expressed Ndt80 first accumulates in an unmodified state early in sporulation (332). Subsequent modification is soon detected, which has been shown to be dependent on Ime2 activity (19, 332). Ime2 has actually been shown to have the ability to phosphorylate Ndt80 *in vitro*. Also, experiments using an analog-sensitive allele of *IME2* (*ime2-as1*) have demonstrated that persistent Ime2 activity is needed for full expression of middle-sporulation genes (19). The mechanism by which Ime2 influences Ndt80 activity has been investigated, however contradictory results have emerged from these studies. The phosphorylation of Ndt80 by Ime2 was found to promote its activity *in vivo* by encouraging efficient interactions between Ndt80 and its MSE binding site (332). This was determined by chromatin immunoprecipitation assays, which demonstrated that Ndt80 binding to promoter DNA was reduced in *ime2* mutants. In contrast to this result, *in vitro* Ndt80 DNA binding assays have shown that phosphorylation of Ndt80 by Ime2 has little effect on DNA-binding activity (333). Though this *in vitro* result does seem to counter the *in vivo* data, it may actually suggest that Ime2 may be promoting Ndt80 binding to chromatin or effecting Ndt80's interaction with other potential transcriptional co-factors. Evidence also exists to suggest that Ime2 may promote full Ndt80 activity by affecting its transcriptional expression. It has been reported that Ime2 may induce *NDT80* expression by eliminating Sum1-mediated repression (268). Although direct modification of Sum1 by Ime2 was not demonstrated, deletion of *SUM1* in an *ime2* strain restores significant expression of *NDT80*.

Both phosphorylation of Ndt80 and regulation of Sum1 seem to be important for Ndt80 activity, which is specifically demonstrated with respect to the pachytene checkpoint. During a pachytene checkpoint-mediated arrest, it has been shown that the Ndt80 protein is both less abundant and less extensively phosphorylated (352). It has also been shown that the pachytene checkpoint requires Sum1 (195). Sum1 protein is stable in *dmc1* mutants which have initiated the pachytene arrest. Also, *sum1* mutants have the ability to partially bypass the block in nuclear divisions, and when combined with the *rad17* mutation, these cells completely bypass the pachytene arrest. Ime2 activity has been found to be biphasic in wild type cells, with peaks corresponding to DNA replication and the meiotic divisions (19). Cells deleted for *ndt80* fail to show the second peak in Ime2 activity, indicating that Ime2 activity is lower in pachytene arrested cells. Put together, these data strongly support the requirement for Ime2 activity towards Ndt80 and Sum1 for the progression from Prophase I into the first meiotic division. This data also emphasizes the central role of the meiosis-specific transcription factor Ndt80 in middle sporulation. Figure I-6 outlines the regulatory network leading to the activation of Ndt80 and middle sporulation gene expression.

MI/MII – The Meiotic Nuclear Divisions: The need for B-type cyclin-CDK (MPF) activity in promoting the meiotic nuclear divisions has been well established. As with mitosis, very little is known about the role that B-type cyclin-CDK activity plays during the meiotic nuclear divisions. Nevertheless, studies of *CLB* mutants in budding yeast have shown that Clb-Cdc28 activity is essential for efficient regulation of MI and MII (60, 109). Certain aspects of chromosomal regulation that the Clbs are believed to regulate in mitosis may also apply during meiosis, such as the spindle dynamics and kinetochore-spindle interactions. However, dramatic differences in the features of chromosome segregation during meiosis may indicate that these mechanisms are regulated much differently in sporulation. Reorganization of the mitotic cell cycle to suit meiosis is distinctly reflected in the differential expression of *CLB* genes observed between these two processes (109). Unlike mitosis, where the *CLB*s are expressed in pairs and sequentially throughout the cell cycle, expression of *CLB*s during meiosis is dramatically altered. A strong, coincident accumulation of mRNA and protein for *CLB1, CLB3, CLB4, CLB5 and CLB6* is observed 4 to 5 hours post meiotic induction (47, 109). Based on the presence of putative MSE sequences in their promoters, and their dramatically reduced expression in *ndt80* mutants, Ndt80 is believed to directly mediate this peak *CLB* transcription (47). Also, unlike the strong *CLB2* expression seen in mitosis, this cyclin is not significantly expressed during meiosis. This feature is particularly interesting, since it is well known that Clb2 appears to be the most important Clb during mitosis (299, 342). Studies done on *CLB* mutants in sporulating cells have found, instead, that *CLB1* seems to be the principle cyclin functioning during the meiotic nuclear divisions (60, 109). Cells mutated for *clb1*, alone or in combination with *clb3* and/or *clb4*, produce the most noticeable defects in sporulation efficiency (109). Defects seen in *clb3, clb4* and *clb3 clb4* cells were less severe than those for *clb1* mutants, demonstrating that Clb1 may be playing a more dominant role during meiosis. *clb2* mutants, on the other hand, show no problems in sporulation, consistent with the lack of expression during meiosis (60, 109). Interestingly, even though the sporulation efficiency, relative to tetrad formation, in *clb1 clb3 clb4* mutants was dramatically reduced, it was shown that spore viability in this strain was significantly high (~90%) (60). It was also discovered that spores seen in dyad asci from *clb1 clb3* and *clb1 clb4* mutants maintained a diploid genome content. These mutants failed to go through MII, indicating that Clb1, Clb3 and Clb4 are important for the transition into MI and crucial for the progression through MII. Due to the essential requirement for *CLB5* and *CLB6* in premeiotic DNA replication, roles for these cyclins in MI and MII prove difficult to determine. Nevertheless, a role for Clb5 and Clb6 in regulating meiotic chromosome division remains a possibility.

Though little is known about the exact function B-type cyclin-CDKs serve during meiotic nuclear divisions, a connection between Cyclin B-Cdc2 and the Auroroa-A kinase in *Xenopus* oocytes has been reported (222). Aurora kinases are a family of Ser/Thr kinases that are conserved from yeast to humans, and are essential for the successful execution of cell division in mitosis (57). Aurora kinases play key roles in regulating the kinetochore and the formation of bipolar spindle, accurate chromosomal segregations and cytokinesis (77). Deletion of the budding yeast Aurora kinase *IPL1* is lethal, and *ipl1* point mutations cause abnormal chromosome segregation leading to aneuploidy (41, 94). Though a role for Ipl1 in meiosis has not been reported, *Xenopus* Aurora-A (Eg2) has been shown to be involved in meiotic maturation in activated oocytes (57). Recently, it has been shown that *Xenopus* Aurora-A kinase activity depends on cyclin B-Cdc2 activity, and cyclin B-Cdc2 has the ability to phosphorylate and activate Aurora-A *in vitro* (222). Direct regulation of Aurora kinases may be an important activity for B-type cyclin-CDKs during meiosis, and Clb-Cdc28 kinases my also modulate Ipl1 in a similar manner in yeast.

As is the case in mitosis, meiotic chromosome segregations require the activation of the APC (151). It has been shown that Clb-Cdc28 activity may be involved in regulating the activity of the APC during meiosis I. APC^{Ama1}, which is specifically inactivated by Mnd2 in meiosis, is also believed to be inhibited by Clb1-Cdc28 activity (263, 275). APC^{Cdc20} in meiosis is thought to be regulated by the spindle checkpoint, becoming activated upon proper attachment of chromosomes to the spindle (246, 390). Although activation of APC^{Cdc20} by Clb-Cdc28 has not been described in meiosis, once APC^{Cdc20} is active, Clb1 degradation ensues, possibly relieving some inhibition on APC^{Ama1} (151). Phosphorylation of Mnd2 by an unknown kinase is believed to relieve the Mnd2-mediated repression of APC^{Ama1}, thereby fully activating this ubiquitin ligase (263, 275). Together, APC^{Ama1} and APC^{Cdc20} can work to promote the meiotic chromosome segregations.

Though similar mechanisms are involved in mitosis and meiosis, the regulation of chromosome division in meiosis must be reorganized to accommodate two consecutive segregation events. Specifically, bipolar attachments of homologous pairs must be established and sister chromatid cohesion must be maintained after the separation of homologous pairs at MI. One aspect of this reorganization is the incorporation of meiosis-specific components that respond to the differential regulation during meiotic progression. As in mitosis, APC-mediated proteolysis of Pds1 (securin) allows Esp1 (separase) to cleave cohesin in meiosis. During MI, loss of cohesion along chromosome arms destroys the linkages between homologous pairs, allowing them to separate to opposite poles (214). However, cohesion between sister chromatids is maintained around the centromeric regions, which is necessary to ensure a successful reductional division. This preservation of cohesion between sister chromatids is due to a variation in the cohesin complex seen in meiosis. Scc1 of the mitotic cohesin complex is replaced with a meiosis specific variant, Rec8 (172). Separase cleavage of Rec8 is seen in chromosome arms during MI, but not around centromeres until MII. Protection of Rec8 in centromeric regions during MI has been attributed to differences in regulation of Rec8 compared to Scc1, and possible structural differences in the cohesin complex in meiosis versus mitosis. For example, phosphorylation of Scc1 by the Polo-like kinase Cdc5 promotes Scc1 cleavage, but this modification is not essential (5). However, Rec8 cleavage absolutely depends on Cdc5 activity (49, 187). It has been suggested that centromeric Rec8 may be protected from Cdc5 modification by Sgo1, a member of the Shugoshin family (171, 216, 293). Sgo1 associates with kinetochores from meiotic-G_2 until MII, and appears to prevents separase-dependent cleavage of Rec8 during MI.

Both mitotic and meiotic chromosome division events require a decrease in CDK activity. In budding yeast, Clb-Cdc28 activity diminishes during the MI/MII transition (37, 215). The protein phosphatase Cdc14, which works to inactivate Clb-Cdc28 during late mitosis, also functions during the MI/MII transition to promote exit from MI. However, residual Clb-Cdc28 activity is required to prevent DNA replication after MI, and this is achieved by opposing regulations balancing the positive and negative influences on Cdc28 activity (214). Increased synthesis of Clb cyclins during MI and MII, together with partial inhibition of APC activity, activation of the Cdc14 phosphatase, and the inactivation of the inhibitory kinase Swe1 maintain a residual level of Clb-Cdc28 activity during the MI/MII transition. However, the mechanisms leading to reactivation of CDK activity during MII are not known. MII chromosome segregation closely resembles mitotic chromosome segregation, where sister chromatids become bi-oriented and are separated. Upon the onset of anaphase II, Esp1 is reactivated, promoting the final meiotic division and resulting in segregation of the nuclear content into haploid complements.

Mid-late and Late Sporulation: About 66 genes are expressed during the mid-late and late stages of sporulation in budding yeast (46). Many of these genes are involved in spore formation. Other genes may be general stress response genes, as well as haploid-specific genes induced due to the reaffirmation of the haploid state within the developing spores. In accordance with a transcriptional cascade, the expression of this gene class is absolutely dependent on Ndt80 activity (46). In fact, over half of these genes have putative MSEs in their promoters, and many of them are probably targets of Ndt80, and possibly repressed by Sum1. However, these genes are expressed distinctly later than the middle sporulation class of genes. This has been attributed to the presence of negative regulatory elements (NREs) in the promoters of these genes that prevent expression during middle sporulation. An example of such repression is seen in the mid-late genes *DIT1* and *DIT2* (95). *DIT1* and *DIT2* are divergently transcribed genes that encode enzymes involved in the synthesis of the dityrosine precursor required for the outermost layer of the spore wall. The NRE^{DIT} has been shown to be located between the start sites of these genes, and represses their expression in an orientation and position-independent manner. Repression at this NRE^{DIT} requires the Ssn-Tup1 repression complex. A more thorough dissection of the NRE^{DIT} has uncovered an 11-base pair palindromic sequence termed the *DIT* repressor element (DRE), as well as an MSE-like sequence (21). It has been demonstrated that repression of *DIT1* and *DIT2* in vegetative cells requires both the DRE and the MSE-like sequence, however, it has not been determined if Sum1 functions in this repression.

Initial steps of spore morphogenesis involve the formation of prospore membranes from vesicles, which are recruited to the cyplasmic sides of each of the four spindle-pole bodies (SPBs) involved in the chromosome divisions (249). The prospore membranes, which are double-membranes, expand during MII, enveloping the nuclear lobes forming upon chromosome segregation. After complete nuclear division, each prospore membrane closes off, fully encapsulating each haploid daughter nucleus. Maturation of the spores occurs via synthesis of a spore wall, which begins in the lumen between the prospore membrane. Eventually, the outer prospore membrane breaks down, and the resulting spore wall is established, providing resiliency towards environmental stresses that the spore may encounter. The final step in this process is the transformation of the intact annucleate mother cell into an ascus housing the four haploid spores into a tetrad.

A Last Look at Meiosis: A distinct transcriptional cascade of gene expression characterizes the sporulation program in budding yeast, which follows the basic fundamental mechanism seen in mitotically proliferating cells. That is, key regulators in one stage of the process regulate events in that particular stage, and also promote the transition from the present stage to the next. Figure I-7 presents the basic schematic of meiosis is *S. cerevisiae*, diagramming the progression through the sporulation program and outlining the key players involved. The study of meiosis in yeast provides an important model for characterizing the regulation of cellular processes seen during a developmental program in eukaryotic cells. The study of sporulation is also intriguing due to the fact that striking parallels exist between the meiotic development of spores and spermatogenesis in higher eukaryotes, offering a useful platform for comparative analyses.

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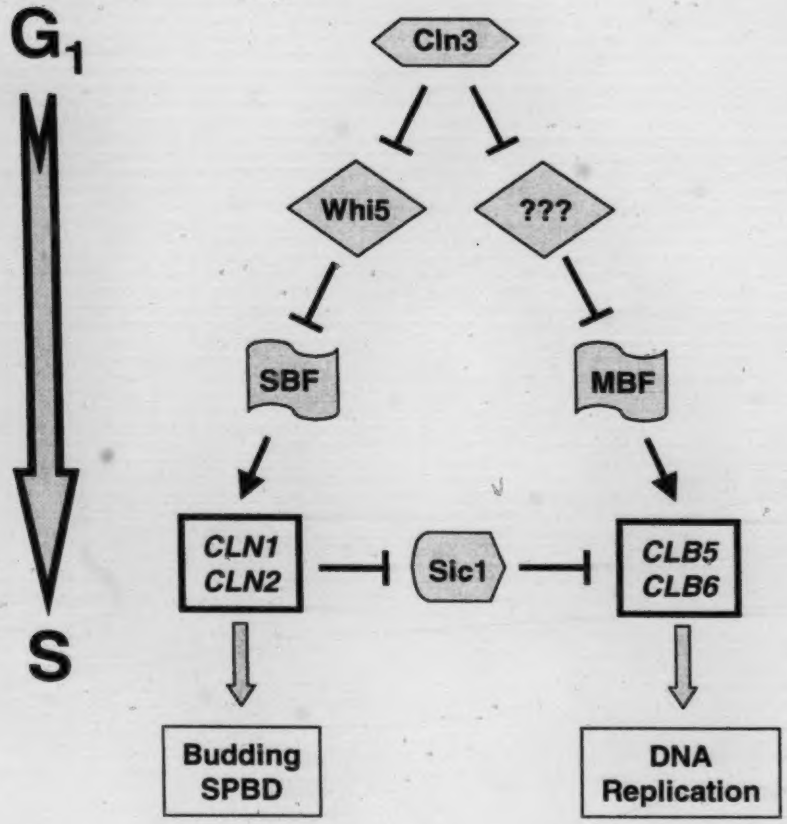
G1
S
Cln3
Whi5
???
SBF
MBF
CLN1
CLN2
Sic1
CLB5
CLB6
Budding
SPBD
DNA
Replication

Figure I-1: START and the G_1/S transition. In *S. cerevisiae*, progression through START promotes the transition from G_1-phase into mitotic S-phase by initiating a hierarchical network of regulatory interactions. This network is prompted by the accumulation of Cln3-Cdc28 activity, which activates a wave of G_1/S transcription mediated by the START-specific transcription factors SBF and MBF. Critical targets of SBF and MBF are the cyclin genes *CLN1*, *CLN2*, *CLB5* and *CLB6*, whose encoded proteins play pivotal roles in coordinating the early events of cell division, such as DNA replication, bud emergence and spindle pole body duplication (SPBD).

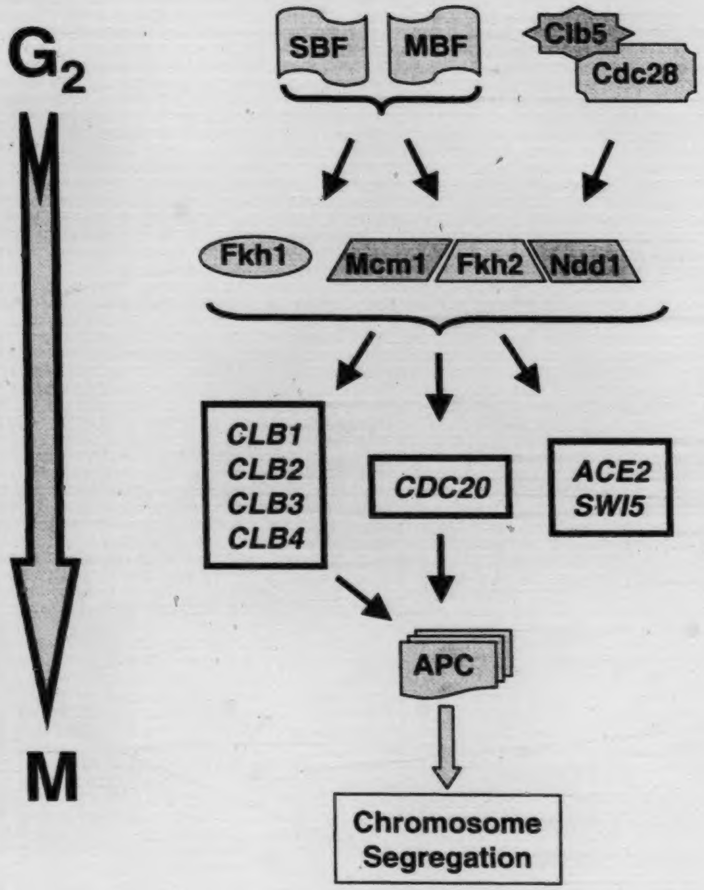
G2
SBF
MBF
Clb5
Cdc28
Fkh1
Mcm1
Fkh2
Ndd1
CLB1
CLB2
CLB3
CLB4
CDC20
ACE2
SWI5
APC
Chromosome Segregation
M

Figure I-2: The G_2/M transition and "Clb2-cluster" genes. Another network of regulatory interactions ensues during G_2-phase characterized by a second wave of transcription, which includes expression of the Clb2-cluster genes. This transition is initiated by SBF and MBF, transcription factors which were activated during START. Together with Clb5-Cdc28, these factors promote the activity of the G_2 transcription factors Mcm1/Fkh2/Ndd1 and Fkh1. Important targets of these G_2 transcription factors (such as *CLB1-4*) promote the onset of M-phase by regulating chromosome segregation. Other important targets of the G_2 transcription factors are *ACE2* and *SWI2*, genes that encode transcription factors required for the M/G_1 transition.

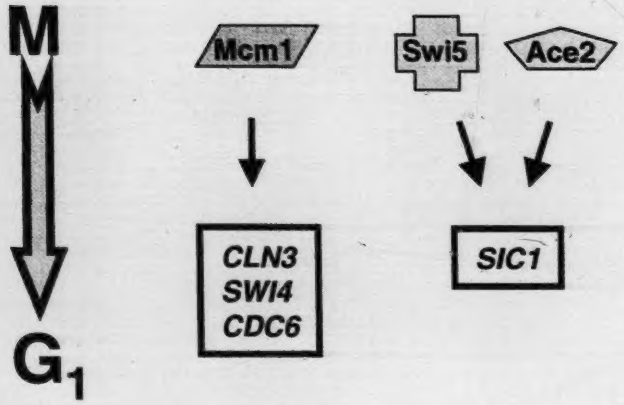

Figure I-3: The M/G_1 transition. A final wave of transcription has been characterized during the exit from mitosis, involving the M/G_1 genes. The transcription factors Swi5 and Ace2, expressed during G_2/M, as well as the Mcm1 transcription factor, work together to express genes required during early G_1. Important M/G_1 genes expressed at this stage are the Cki gene *SIC1*, the transcription factor *SWI4*, the G_1-cyclin *CLN3*, and the replication protein *CDC6*.

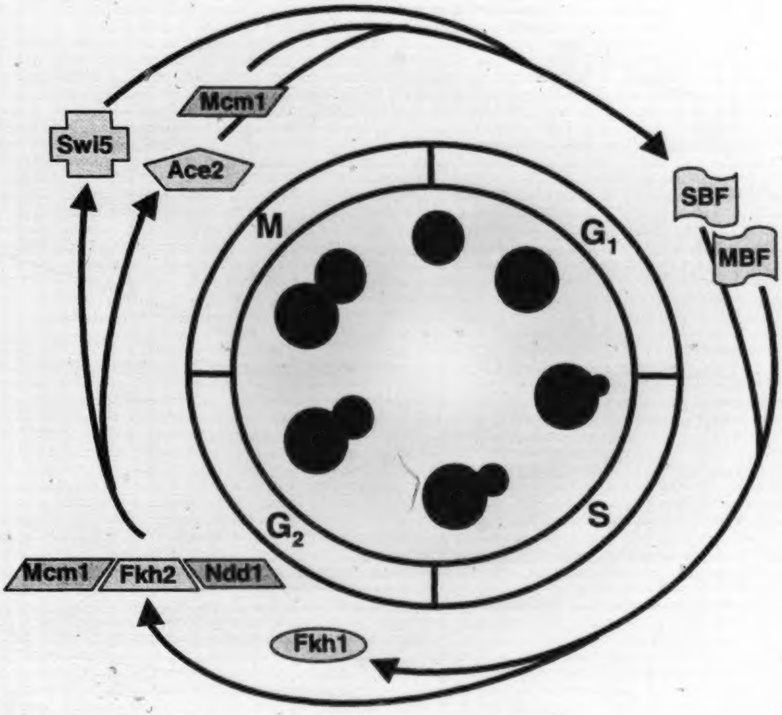

Figure I-4: A transcriptional cascade drives the cell division cycle. Serial regulation of transcriptional regulators proves to be the underlying mechanism advancing the cell cycle in *S. cerevisiae*. Activation of transcription factors in one stage of the cell cycle, serve to regulate events of that stage, but also lead to the activation of transcription factors required in the following stage. This mechanism consequently initiates a cascade of gene expression driving the process of cell division. This figure has been adapted from Simon et al. (2001) (324).

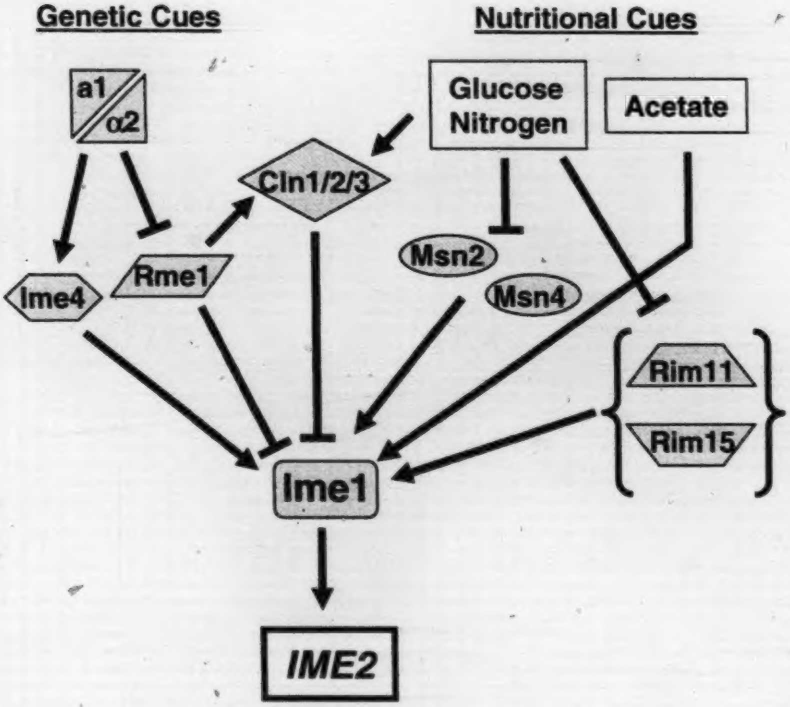
Genetic Cues
Nutritional Cues
a1
α2
Glucose
Nitrogen
Acetate
Cln1/2/3
Ime4
Rme1
Msn2
Msn4
Rim11
Rim15
Ime1
IME2

Figure I-5: Activation of the "Master Regulator" of meiotic development, Ime1. Induction of meiotic development in *S. cerevisiae* relies on appropriate environmental and genetic cues, such as nutritional status and the diploid state. Regulatory pathways initiated by these stimuli converge on a key factor in sporulation, the meiosis-specific transcription factor Ime1. Both transcriptional and post-transcriptional mechanisms affect the activity of Ime1, resulting in a strictly coordinated induction of the meiotic process. Once activated, Ime1 promotes the expression of early meiotic genes, most notably *IME2*, which encodes a meiosis-specific protein kinase essential for meiotic development.

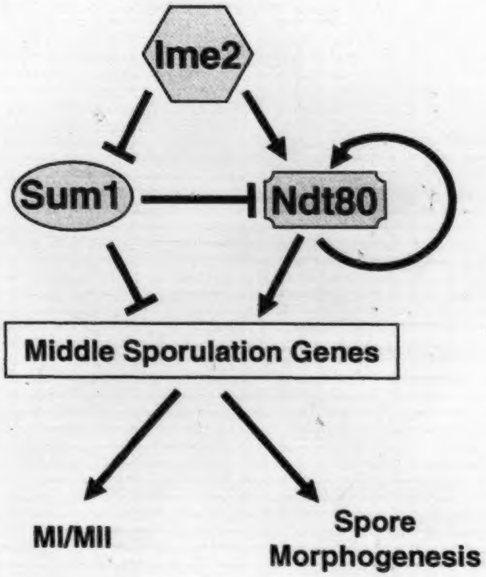

Figure I-6: The coordinated regulation of middle sporulation. The transition into middle sporulation in *S. cerevisiae* is a tightly regulated process, falling under strict checkpoint control. Middle sporulation is characterized by a wave of gene expression regulated by the meiosis-specific transcription factor Ndt80. This transcription factor appears to be the central target of this checkpoint control, however the precise nature of this regulation remains unknown. It is possible that the coordinated efforts of Ime2 (activation) and Sum1 (repression) are employed to mediate Ndt80 activity and the onset of middle sporulation. Once activated, Ndt80 promotes the expression of middle sporulation genes, promoting the meiotic nuclear divisions and spore morphogenesis.

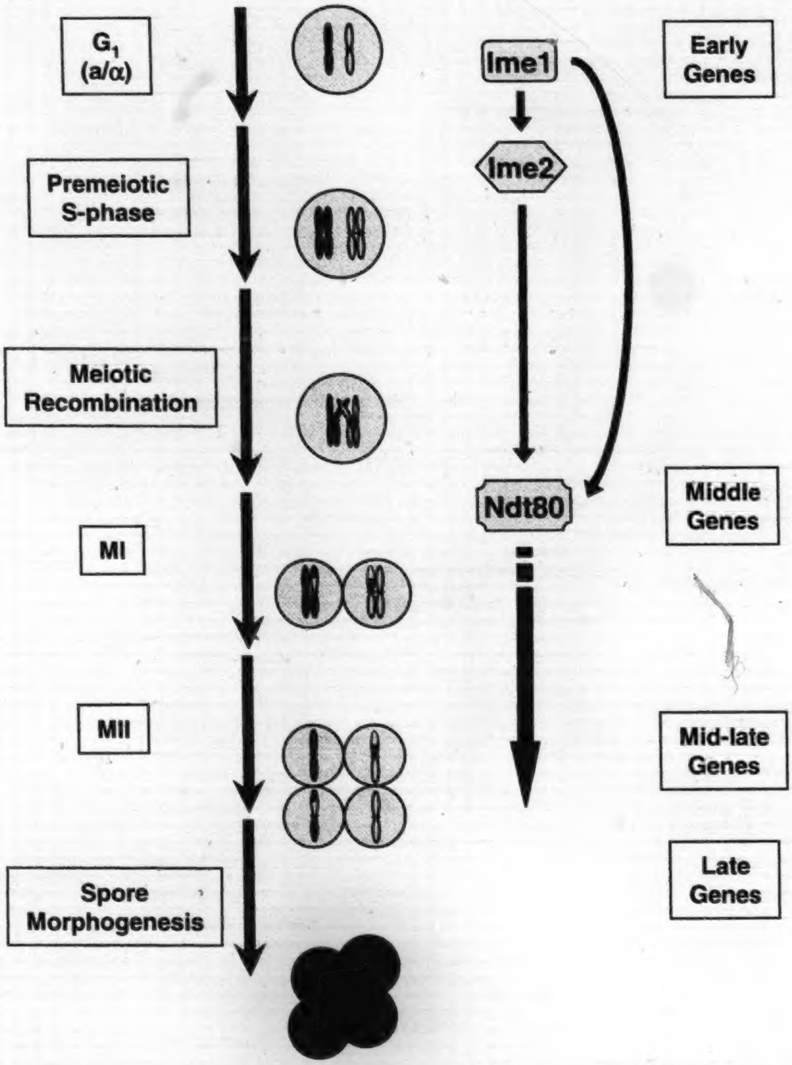
G1 (a/α)
Premeiotic S-phase
Meiotic Recombination
MI
MII
Spore Morphogenesis
Ime1
Ime2
Ndt80
Early Genes
Middle Genes
Mid-late Genes
Late Genes

Figure I-7: An overview of sporulation in *Saccharomyces cerevisiae*. Meiotic development in budding yeast involves the coordination of meiotic chromosome division and spore morphogenesis, ultimately leading to the formation of an ascus housing a tetrad of haploid spores. This process can be separated into at least 4 distinct temporal stages – early, middle, mid-late and late. Each stage is characterized by a wave of gene expression, which is tightly regulated by a cascade of transcription factors. These transcription factors serve as central regulators for meiotic development coordinating the key transitions in this process. A dramatic alteration of the cell division cycle, and reorganization of the gene regulatory network makes sporulation in *S. cerevisiae* an exceptional model for studying cellular development.

Chapter II

Materials and Methods

II.1 – The Long Road

Throughout the course of the graduate work presented in this thesis, a wide range of genetic, molecular and biochemical techniques were employed to facilitate a thorough investigation of the related field of study. For all experiments reported herein, a considerable degree of meticulousness was exercised (eg. appropriate controls and careful technique) to ensure accurate analysis of the data. Also, great care has been taken to confirm reproducibility of the data, and to ensure unbiased interpretation of our results. The following chapter details the techniques utilized for the presented work, and will serve as a reference of materials and methods for the entire thesis.

II.2 – Strains and Growth Conditions

All yeast strains utilized in this study conform to the SK1 genetic background (3). These strains were derived from DSY1030 (*MAT*a *lys2 ho::LYS2 ura3 leu2::hisG trp1::hisG arg4Bgl his4X*) and DSY1031 (*MAT*α *lys2 ho::LYS2 ura3 leu2::hisG trp1::hisG arg4Nsp his4B*). Table II-1 details all the strains used in this study with their relevant genotypes. All strains were generated using standard genetic techniques (5). The [illegible]::Kan^{R}, *clb6*::*TRP1*, *swi4*::*LEU2*, *mbp1*::*URA3*, *ndt80*::Kan^{R} deletions, and the Ndt80-HA tagged construct have been described previously (6, 8-10). The Mbp1-Myc tagged construct was generated by amplification of the 3'-end of *MBP1*, including the C-terminus of the open reading frame. This was ligated into the yeast integration vector YIplac204, followed by an in-frame insertion of a cassette encoding 15 copies of the c-Myc epitope. This tagged construct was then introduced into DSY1030 (*MAT*a) and DSY1031 (*MAT*α), with insertion directed towards the C-terminus of *MBP1*. Haploids were then mated, and diploids homozygous for the tagged allele were isolated. *SWI4* in DSY1030 (*MAT*a) and DSY1031 (*MAT*α) was C-terminally tagged with three copies of the HA epitope using the vector p*URA-SWI4HA* as described (1). Haploids were then mated, and diploids homozygous for the tagged allele were collected. Isolation of the *CLB5* open reading frame was accomplished by purifying a *BamHI-SacI* fragment encompassing this gene. This fragment was placed under the regulation of 900bp of *IME2* promoter sequence to create *IME2-CLB5*, or 800bp of *MET3* promoter sequence to create *MET3-CLB5*. Both these constructs were then individually integrated at the *URA3* locus into the *clb5 clb6* haploid strains DSY1064 (*MAT*a) and DSY1065 (*MAT*α). Appropriate diploid for each of these strains were created by mating.

For creation of the *CLB5* promoter constructs, a 2.6kb *XhoI-SacI* fragment containing the *CLB5* open reading frame and 400bp of promoter sequence was ligated into the yeast integration vector YIplac211. Six tandem copies of the HA epitope were introduced at the C-terminus of this *CLB5* construct. Also, an *XhoI* fragment encompassing an additional 590bp of promoter sequence was introduced upstream of the 400bp promoter sequence already present. Therefore, this *CLB5* promoter construct contains 990bp of total upstream promoter sequence relative to the initiation codon, and provides sufficient regulation of the *CLB5* gene.

Creation of the non-functional *clb5* reporter gene was accomplished by deletion of a 250bp *BspE1-Bsu36I* fragment within the *CLB5* open reading frame from the construct described above; also the transcriptional stop sequence within this construct was altered to allow us to distinguish the *clb5* transcript relative to the endogenous *CLB5* mRNA.

MCB mutations within the *CLB5* promoter sequence (Chapter IV) were generated by site-directed mutagenesis using the Altered Sites® *in vitro* Mutagenesis System (Promega) as described previously (8). This system involves an oligonucleotide-directed mechanism to introduce site-specific mutations into the gene of interest. Briefly, the *CLB5* construct described above (*Xho1-Sac1* fragment) was subcloned into the vector pALTER®-1 (Promega) and single-stranded DNA was prepared using the helper phage M13KO7 (Promega) as described (11). pALTER®-1 (Promega) contains genes for ampicillin and tetracycline resistance, which allows for antibiotic selection to facilitate high frequency isolation of mutants. The ampicillin resistance gene is inactivated, but resistance can be restored by using the Ampicillin Repair Oligonucleotide (supplied by manufacturer), which repairs the inactive gene on the mutated strand during the mutagenesis reaction. Therefore, appropriate oligonucleotides corresponding to the targeted MCBs within the *CLB5* promoter (synthesized by Sigma) were annealed simultaneously with the repair oligonucleotide, then the mutant strand was synthesized using T4 DNA polymerase and T4 DNA Ligase (both supplied by the manufacturer). This reaction was then transformed into a repair minus strain of *E. coli* (ES1301 *mutS*), to propagate the desired mutations. Plasmid DNA was purified, transformed into the *E. coli* strain DH5α, and selected with ampicillin for the mutated vectors. Finally, recovered MCB mutant constructs were subcloned back into YIplac211, then sequenced to confirm the incorporation of the desired mutations and to ensure that no unintended mutations had been generated. Figure II-1 diagrams the schematic of the *CLB5* promoter and outlines all the MCB mutations generated.

Mutations in MSE1 and MSE2 within the *CLB5* promoter (Chapter IV and V) were generated by PCR mediated mutagenesis, involving sequence splicing and overlap extension as described in Horton et al. (1989) (12). Basically, complementary primers were created (Sigma) which included the desired MSE mutations. These primers were used in separate reactions with the appropriate upstream or downstream primer to amplify sequence 5' and 3' of the targeted MSE respectively. These products were then used together in another PCR reaction to extend the annealed overlapping complementary sequences. Finally, this extended product was amplified with the upstream and downstream primers, and then subcloned into the appropriate region within the *CLB5* promoter. As with the MCB constructs, these MSE constructs were also sequenced to confirm the incorporation of the desired mutations, and also to ensure that no unintended mutations had been generated. Figure II-1 diagrams the schematic of the *CLB5* promoter and outlines all the MSE mutations generated. PCR mediated mutagenesis (12) was also used to generate the Δ178 and Δ179 deletion constructs as well (Chapter IV). Basically, primers with overlapping sequence that facilitated the deletion of 100bp (Δ178) or 176bp (Δ179) within the *CLB5* promoter were used in the protocol described above.

The promoter construct that places *CLB5* under the regulation of only two MCB sequences (MCB-*CLB5*) was created by annealing the following oligonucleotides:

WTKXF 5'- CTCTCGAGTGAAGACGCGCCCTTGATGGC- 3' and

WTKXR 5'- TCGAGCCATCAAGGGCGCGTCTTCACTCGAGAGGTAC - 3'

and inserting this duplex into the Δ179 promoter so that the oligonucleotide replaces the 176bp deletion in this construct (Chapter IV). The construct that places *CLB5* under the regulation of one mutant and one wild-type MCB (mcbx-*CLB5*) was created in a similar manner, however the oligonucleotides used encoded a mutant MCB sequence in which the core CGCG had been changed to CGAG.

Those constructs expressing the *clb5* reporter gene were integrated at the *URA3* locus in the wild type *CLB5 CLB6* haploids DSY1030 (*MAT*a) and DSY1031 (*MAT*α). Those constructs expressing the *CLB5* open reading frame were integrated at the *URA3* locus in the *clb5 clb6* mutant haploids DSY1064 (*MAT*a) and DSY1065 (*MAT*α). Single-copy integration of these constructs was confirmed by Southern blot analysis of genomic DNA purified from the transformed haploid strains (see below for Southern blot protocol).

Generation of *clb5 clb6 mbp1* MCB-*CLB5* or *clb5 clb6 mbp1* mcbx-*CLB5* strains (Chapter IV) involved crossing the *clb5 clb6* MCB-*CLB5* and *clb5 clb6* mcbx-*CLB5* haploids (*MAT*a) to an *mbp1*::Kan^R strain (DSY1475, *MAT*α). The resulting diploids were sporulated, tetrads were dissected, and colonies derived from the spores that were *clb5*::Kan^R *clb6::TRP1 mbp1*::Kan^R *URA3*::MCB-*CLB5* and *clb5*::Kan^R *clb6::TRP1 mbp1*::Kan^R *URA3*::mcbx-*CLB5* were identified. Appropriate haploids were crossed to make homozygous strains for each construct.

For creation of the *lacZ* reporter genes driven by the *CLB5* promoter (Chapter V), various *CLB5* promoter fragments were created via PCR amplification of either the wild-type, Δ*mse1*, Δ*mse2* or Δ*mse1*Δ*mse2* promoter constructs. Upstream primers used in this amplification included the *Spe1* restriction site and downstream primers included the *Sal1* restriction site. Amplified promoter fragments were purified, digested with *Spe1* and *Sal1*, and then ligated upstream of the *lacZ* reporter gene in YEplac195-*lacZ* using *Xho1* and *Xba1* compatible ends; this vector contains the *lacZ* gene under the regulation of *CYC1* TATA box. The promoters of all these reporter gene constructs were sequenced to confirm the desired mutations and to ensure that no inadvertent mutations were generated. These *lacZ* reporter constructs were transformed into diploid wild type cells (DSY1089, MATa/α) and diploid *ndt80* cells (DSY1150, *MAT*a/α).

Growth of yeast strains in this study were carried out in rich YEP (1% yeast extract, 2% peptone, 30mg/litre adenine 30mg/litre tryptophan) supplemented with 2% dextrose (YEPD) at 30°C. Selection media lacking uracil (-ura; 0.02g/L Ade and 0.1g/L of the following amino acids – A, R, N, C, Q, E, G, H, I, L, K, M, F, P, S, T, W, Y, V) supplemented with 2% dextrose, 1.7g/L Difco™ yeast nitrogen base (Becton, Dickinson and Co.), and 5g/L $(NH_4)_2SO_4$, was used when required to maintain selection for the *lacZ* reporter gene constructs. To achieve synchronized meiotic induction sporulation experiments were performed as described previously (9). Briefly, strains were grown on rich glycerol agar (YEPGlyc; 3% Glycerol) to select for mitochondrial function, then grown overnight in rich YEPD medium. Cultures were then diluted to $1\text{-}2\times10^7$ and pre-grown in rich YEP supplemented with 2% potassium acetate (YEPKAc) for 12-14 hours. Cells were then washed once with sterile de-ionized water and inoculated into sporulation medium (SPM, 1% potassium acetate in de-ionized water). Sporulation was carried out under vigorous agitation at 30°C. For sporulation time-course experiments, which were carried out extensively in the studies outlined in the following chapters, cells were collected from sporulating cultures every hour up to 12 hours. Cells from each sample were pelleted and stored at -80°C, or fixed in 70% ethanol for flow cytometry analysis.

II.3 – RNA Extraction

Total RNA was isolated from frozen cell pellets as described previously (8). Briefly, pellets were vortexed for 10-15 minutes (at 4°C) with glass beads in 20mM Tris-HCl (pH 8.0), 500mM NaCl, 10mM EDTA (ethylenediaminetetraacetic acid), 1% sodium dodecyl sulfate (SDS), and an equal volume of water-saturated phenol (acid phenol). Following the vortexing, phases were separated by high speed centrifugation and the aqueous phase was recovered. This aqueous phase was subjected to two more extractions with acid phenol containing 10% 3M sodium acetate (pH 4.0), then extracted once with chloroform. The RNA samples (aqueous phase) were then recovered and precipitated with two volumes of 95% ethanol. Finally, RNA pellets were resuspended and stored in 60% ethanol at -20°C. Quantitation of RNA samples for use in Northern blot and RT-PCR analyses (described below) was performed by measuring A_{260}.

II.4 – Northern Blot Analysis

Extraction of total RNA was performed as described above. For Northern blot analysis of sporulation time course experiments, 20µg of total RNA from each sample was separated on a 1.2% agarose gel containing 1X-MOPS buffer (40mM MOPS [3-(N-Morpholino) Propane Sulfonic acid], 17mM NaOH, 5mM sodium acetate, 1mM EDTA) and 7% Formaldehyde, and electrophoresed in 1X-MOPS buffer. Separated RNA was then transferred to a MagnaGraph nylon membrane (0.45 Micron, Osmonics Inc.), which was then pre-hybridized for 2-3 hours in N-Hyb Solution (4M NaCl, 100mM Na_2HPO_4/NaH_2PO_4 (pH 6.5), 4mM EDTA, 0.2% SDS, 10% Dextran Sulfate) at 65°C, then hybridized overnight with radiolabeled probe (prepared probe was denatured and added directly to the pre-hybridization solution). Northern blots were probed for *CLB5* using a 1.5kb *AflII* fragment which encompassed most of the *CLB5* open reading frame. *NDT80* was probed with a 1.6kb *BglII/SalI* fragment encompassing the *NDT80* open reading frame. Detection of *SPS1* and *SPS2* was accomplished by probing with a 3kb *ClaI* fragment from plasmid p18 (4). *TMP1* and *RNR1* were detected with PCR fragments amplified from the open reading frame of their respective genes. *ACT1* transcripts were probed with a 1.6kb *Bam*HI-*Hin*dIII containing the *ACT1* gene. DNA probes were labeled with [α-^{32}P]dCTP using the Random Primers DNA Labeling System (Invitrogen). Northern blot signals were visualized and quantified using a Molecular Dynamics (Sunnyvale, CA) STORM phosphorimager.

II.5 – Reverse Transcriptase-PCR and Southern Blot Analysis

Extraction of total RNA was performed as described above. 20µg of total RNA was then treated with the TURBO DNA-*free*™ system (Ambion) to remove contaminating DNA from the preparation. 5µg of DNaseI-treated RNA was tested using PCR for ensure complete removal of contaminating DNA. 2µg of purified total RNA was subjected to reverse-transcription (SuperScript™ III Reverse Transcriptase, Invitrogen) using gene specific RT primers (see Chapter V, Table V-1), targeting *CLB5*, *lacZ*, and *ACT1* transcripts for cDNA synthesis. Then 2µl of the RT reaction was used in PCR to amplify the cDNAs created, aiming for the linear range of synthesis to ensure quantitative results. To determine the PCR conditions permitting us to target the linear range, we set up control reactions containing fixed amount of primers and plasmid sequence (encoding *lacZ*), and analyzed the synthesis of these reactions throughout the PCR amplification program. Various fixed quantities of primers and plasmid were tested. Optimal quantities and the appropriate number of cycles were determined for linear range analysis. Primers used were designed to create products of distinct sizes. Southern blot analysis was used to quantitatively analyze RT-PCR products. Briefly, RT-PCR samples were eletrophoresed through 1.2% Agarose, and transferred to a MagnaGraph nylon membrane (0.45 Micron, Osmonics Inc.). Pre-hybridization of the membrane was done for 2-3 hours at 55°C in S-Hyb Solution (6X SSC (saline-sodium citrate), 5X Denhardt's reagent, 0.4% SDS, 50µg/ml Salmon Sperm DNA) then blots were hybridized overnight with radiolabeled probe (prepared probes were denatured and added directly to the pre-hybridization solution). *CLB5*, *lacZ* and *ACT1* products were detected using probes created from PCR fragments of these specific open reading frames. Probes were radiolabeled with [α-^{32}P]dCTP via Random Primers DNA Labeling System (Invitrogen). Southern blot signals were visualized and quantified using a Molecular Dynamics (Sunnyvale, CA) STORM phosphorimager.

II.6 – Protein Extraction, Western Blot Analysis and Kinase Assays

Protein samples for Western blot analysis of Ndt80-HA and Mbp1-Myc were prepared by TCA extraction as described (2). Briefly, pelleted cells were resuspended in 20% TCA (trichloroacetic acid). An equal volume of glass beads was added to the suspension, which was vortexed vigorously for 2-4 minutes. Then 400µl of 5% TCA was added and the entire extract was siphoned away from the glass beads into a fresh tube. Samples were subjected to high-speed centrifugation to pellet proteins (14,000rpm for 10 minutes). Supernatant was discarded and the pellet was resuspended in 2X Laemmli Buffer (50mM Tris-HCl (pH 8.5), 4% SDS, 20% glycerol, 0.2% bromophenol blue, 10% β-mercaptoethanol, 200mM dithiothreitol (DTT)) and an equal volume of 50mM Tris-HCl (pH 8.5). Protein samples for Western blot analysis of Swi4-HA were prepared by immunoprecipitation of protein extracts. Briefly, frozen cell pellets were resuspended in Lysis Buffer (50mM Tris-HCl (pH 7.4), 100mM NaCl, 0.1% NP-40, 1mM EDTA, Protease Inhibitors [2µg/ml aprotinin, 2µg/ml leupeptin, 1µg/ml pepstatin A, 100µg/ml phenylmethyl sulfonyl fluoride (PMSF)], and Phosphatase Inhibitors [10mM sodium pyrophosphate, 10mM sodium orthovanadate, 5mM EGTA (ethylene glycol-bis-(b-aminoethyl-ether)-N,N,N',N'-tetra-acetic acid), 5mM EDTA]). Resuspended cells were then vortexed at 4°C with an equal volume of glass beads for 4x1min with intervals of 1 minute on ice between vortexing. Lysates were then extracted away from glass beads and clarified by centrifugation (14,000rpm, for 10min at 4°C). Clarified lysates were diluted with one volume of IP-Buffer (50mM Tris-HCl (pH 7.5), 100mM NaCl, 0.5% Triton X-100, Protease Inhibitors and Phosphatase Inhibitors), then incubated with 1µl of anti-HA Ascites fluid (Babco) for 1 hour at 4°C. Lysates were then further incubated with 20µl of pre-swollen beads for 1 hour (4°C) to capture the immunocomplexed Swi4-HA. Low-speed centrifugation (2000rpm, 3min) was used to collect the beads. Beads were washed 4x with IP-Buffer, 1x with IP-250 Buffer (50mM Tris-HCl (pH 7.5), 250mM NaCl, 0.5% Triton X-100, Phosphatase Inhibitors), then once more with IP Buffer. Protein pellets from TCA extraction and immunoprecipitated protein on beads were resuspended in 2X Laemmli Buffer and an equal volume of 50mM Tris-HCl (pH 8.5), and protein samples were separated by eletrophoresis on 10% SDS-polyacrylamide gels. Separated proteins were transferred to an Immobilon-P transfer membrane (Millipore), and Western blots were probed with monoclonal antibody 12CA5 (Babco) at a dilution of 1:10,000 to detect Swi4-HA and Ndt80-HA, or with monoclonal antibody 9E10 (Babco) at a dilution of 1:10,000 to detect Mbp1-Myc. Blots were also probed with monoclonal antibody anti-PSTAIRE (Sigma) at a dilution of 1:10,000 as a loading control for Ndt80-HA and Mbp1-HA. All of the primary antibodies were detected with horseradish peroxidase conjugated anti-mouse antibodies (Jackson Labs). Immunoprecipitation and Clb5-associated histone H1 kinase assays were preformed as described (9). Clb5-HA was immunoprecipitated from protein lysates as described above. Bead-bound Clb5-associated kinase was then assayed for histone H1 kinase activity as described previously (9). Briefly, bead bound Clb5-HA was washed once with

2X Kinase Buffer (100mM Tris-HCl (pH 7.5), 10mM $MgCl_2$, 2mM DTT, 20μM ATP), then resuspended in 10μl of 2X Kinase Buffer. Histone H1 substrate and [γ-^{32}P]ATP is then added, and reactions are carried out at 30°C for 15-20 minutes. Kinase reactions were then separated on 10% SDS-polyacrylamide gels. After electrophoresis, gels were dried and the phosphorylated substrates were visualized and quantitated using a Molecular Dynamics (Sunnyvale, CA) STORM phosphorimager.

II.7 – Electrophoretic Mobility Shift Assays/DNA Binding

Expression and purification of recombinant Ndt80-6xHIS from transformed *E. coli* BL21-DE3 (Stratagene) has been described in detail previously (7). Briefly, *E. coli* strain BL21-DE3 (Stratagene) transformed with an Ndt80 expression vector was grown overnight at 37°C in LB (Luria-Bertani medium) supplemented with 100μg/ml ampicillin. The overnight culture was then used to inoculate a 1 litre culture of LB containing 100μg/ml ampicillin, and this culture was grown at 37°C to a density (OD_{600}) of approximately 0.5. Once the correct density was achieved, production of recombinant protein was induced with the addition of IPTG (isopropyl β–D-thiogalactosidase) to a final concentration of 1mM. After three hours of induction, the cells were harvested by centrifugation (10 minutes at 4000g, 4°C), and the pellet was stored at -80°C. Frozen pellets were resuspended in 50mM NaH_2PO_4/Na_2HPO_4 (pH 7.6), 500mM NaCl, 10mM imidazole, 1% Triton X-100, and Protease Inhibitors (as described above). The suspended cells were then lysed and sonication was used to shear genomic DNA (4 x 20s pulses at 75% maximal power with a Braun Labsonic-U sonicator fitted with a microtip probe). Clarification of the crude lysate was accomplished via high speed centrifugation (30min at 20,000g, 4°C). Clarified lysate was then applied to a 4ml column packed with $NiSO_4$-charged chelating Sepharose (Amersham-Pharmacia Biotech). The columns were washed with 10 volumes of wash buffer (50mM NaH_2PO_4/Na_2HPO_4 (pH 7.6), 500mM NaCl, 20mM imidazole, 1% Triton X-100). Bound proteins were then eluted with 2 column volumes of elution buffer (50mM NaH_2PO_4/Na_2HPO_4 pH 7.6, 150mM NaCl, 250mM imidazole, and 0.1% Triton X-100). The eluate was collected in 0.5ml fractions and assayed for recombinant Ndt80 by SDS-PAGE. Fractions most highly enriched for Ndt80 were pooled and dialyzed overnight against 50mM NaH_2PO_4/Na_2HPO_4 pH 7.6, 100mM NaCl, 10% glycerol, 1mM DTT (dithiothreitol), and 0.1% Triton X-100. Protein concentration was determined by using bovine serum albumin (BSA) as a protein standard. By creating a dilution series of BSA on Coomasie-stained SDS-polyacrylamide gels, the specific concentration of Ndt80 in our combined fraction was determined. DNA fragments used in Ndt80 binding assays were PCR products derived from the wild type, Δ*mse1*, Δ*mse2* and Δ*mse1* Δ*mse2* promoter constructs described above. Various combinations of primer pairs shown in Table V-1 were used to create an array of DNA fragments representing regions of the *CLB5* promoter. Fragments were end-labeled with [γ-^{32}P]ATP using polynucleotide kinase (Invitrogen), and purified from unincorporated nucleotides using a gel filtration spin-column (Mircro BioSpin 30, Biorad). Binding reactions containing purified Ndt80 and labeled promoter fragments were analyzed. Binding conditions, native polyacrylamide gel electrophoresis and radioactive gel analysis were carried out as previously reported (7). 1pmol of radiolabeled promoter fragment and approximately 14pmol of purified Ndt80 were added to each 20μl binding reaction containing 20mM Tris-HCl (pH 8.0), 5mM $MgCl_2$, 0.1mM EDTA, and 10% glycerol. Ndt80/fragment relative binding affinity was assayed by titration with an increasing amount of non-specific nucleic acid competitor poly dI:dC added to reactions after radiolabeled probe and Ndt80. Fixed amounts of poly dI:dC were used at times for comparisons between fragments. Specific competition of the Ndt80/fragment complex was attained by including 25 fold or 50 fold excess unlabeled oligonucleotide duplex containing a consensus MSE sequence. Binding reactions were carried out at room temperature for 5 minutes then loaded onto 5% native polyacrylamide gels. After electrophoresis, gels were dried and labeled species were visualized and quantitated using a Molecular Dynamics (Sunnyvale, CA) STORM phosphorimager. Due to multiple shifted species seen under most reaction conditions, quantitation of the unbound probe proved to be more reliable for binding analysis.

II.8 – Other Procedures

Microscopic examination of cultures incubated in SPM for 24 hours was performed to assay for completion of sporulation. Two hundred cells in three fields were counted and the percentage of cells forming asci was scored.

Progression through the meiotic chromosomal divisions (MI and MII) was monitored via staining of nuclear DNA using 4',6-diamidino-2-phenylindole (DAPI) as previously described (9). Nuclei were visualized using a Zeiss Axioskop II. Two hundred cells in three fields were counted for each sporulating strain documenting mono-, bi- and tetranucleate cells. Images of yeast cells growing in culture were captured with a Zeiss Axioskop II and a SPOT™ Digital Microscope Camera (Diagnostic Instruments Inc). Nuclear-DNA content of fixed propidium-iodide-stained cells was analyzed by flow cytometry as described previously (9). Briefly, 70% ethanol-fixed cells were rehydrated with 50mM Tris-HCl (pH 8.0), then treated with 1mg/ml RNase A (Sigma) prepared in 50mM Tris-HCl (pH 8.0) for at least 2 hours at 37°C. Following RNase treatment, cells were recovered and resuspended in a 200µl solution containing 5g/L pepsin A (Sigma) and 5ml/L concentrated HCl. Cells were incubated in pepsin solution for 1 hour at 37°C, and then neutralized with 1ml of 50mM Tris-HCl (pH 8.0). Cells were recovered and resuspended in Propidium Iodide Staining Solution (180mM Tris-HCl (pH 7.5), 190mM NaCl, 69mM $MgCl_2 \cdot 6H_20$, 50mg/L propidium iodide) and stained overnight at 4°C. Stained cells were diluted in 2.5ml of 50mM Tris-HCl (pH 8.0), sonicated and then subjected to flow cytometry. Cell volume data from asynchronous growing cultures was collected using a Coulter Z2 particle size analyzer (Hialeah, FL).

II.9 – References

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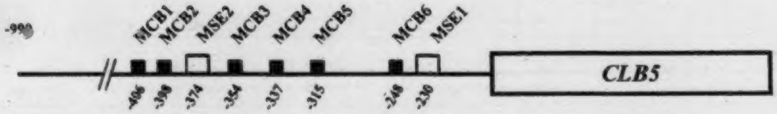
Table II-1

Saccharomyces cerevisiae **strains used**

Strain	Relevant Genotype
DSY1030	*MAT*a
DSY1031	*MATα*
DSY1089	*MAT*a/*MATα*
DSY1064	*MAT*a *clb5*::Kan^R *clb6::TRP1*
DSY1065	*MATα clb5*::Kan^R *clb6::TRP1*
DSY1092	*MAT*a/*MATα clb5*::Kan^R/*clb5*::Kan^R *clb6::TRP1/clb6::TRP1*
DSY1491	*MAT*a/*MATα ime1*::Kan^R/*ime1*::Kan^R
DSY1087	*MAT*a/*MATα ime2::TRP1/ime2::TRP1*
DSY1149	*MAT*a/*MATα clb5*::Kan^R *clb6::TRP1 URA3::IME2-CLB5*
DSY1147	*MAT*a/*MATα clb5*::Kan^R *clb6::TRP1 URA3::MET3-CLB5*
DSY1498	*MAT*a/*MATα swi4::LEU2/swi4::LEU2*
DSY1475	*MAT*a/*MATα mbp1*::Kan^R
DSY1476	*MAT*a/*MATα mbp1*::Kan^R/*mbp1*::Kan^R
DSY1150	*MAT*a/*MATα ndt80*::Kan^R/*ndt80*::Kan^R
DSY1157	*MAT*a/*MATα mbp1::URA3/mbp1::URA3 ndt80*::Kan^R/*ndt80*::Kan^R
DSY1479	*MAT*a/*MATα MBP1-15XMyc/MBP1-15XMyc*
DSY1486	*MAT*a/*MATα SWI4-3XHA/SWI4-3XHA*
DSY1291	*MAT*a/*MATα NDT80-3XHA/NDT80-3XHA*

A



B

MCB Mutations

MCB2 (-398)	GACGCGCC	⟶	GACGAGCC
MCB3 (-354)	GGCGCGTC	⟶	GGACGTTC
MCB4 (-337)	CACGCGCT	⟶	CACGAGCT
MCB5 (-315)	TAGCGCCC	⟶	TAGCATGC
MCB6 (-248)	ACCGCGAA	⟶	ACCTAGAA

C

MSE Mutations

MSE1 (-230)	AACGCAAAT	⟶	GATCCGGCT
MSE2 (-374)	GGTACAAAA	⟶	GGATCCCCA

Figure II-1: Mutagenesis of the *CLB5* promoter. (A) A schematic representation of the *CLB5* promoter is presented, outlining the recognized regulatory elements and their relative positions with respect to the ATG start codon. (B) Site-specific mutagenesis was used to mutate 5 of 6 putative MCBs within the *CLB5* promoter. Listed are the sequences of each MCB along with the mutations introduced (note, underlined is the core CGCG sequence corresponding to the established consensus MCB). (C) PCR-mediated splice-overlap mutagenesis was used to mutate both potential MSEs identified in the *CLB5* promoter. Listed are the recognized MSE sequences along with the mutations incorporated (note, underlined nucleotides highlight the introduced mutations).

Chapter III

The START-Specific Transcription Factor MBF is Functional During Meiotic Development in *Saccharomyces cerevisiae* but Only Influences a Subset of MCB-Regulated Genes

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Sheetal A. Raithatha and David T. Stuart (2005). Meiosis-Specific Regulation of the *Saccharomyces cerevisiae* S-phase Cyclin *CLB5* Is Dependent on *MluI* Cell Cycle Box (MCB) Elements in Its Promoter but Is Independent of MCB-Binding Factor Activity. Genetics 169:1329-1342.

III.1 – Introduction

In *Saccharomyces cerevisiae*, passage through START results in a wave of transcription coordinately mediated by the related transcription factors SBF (SCB-binding factor) and MBF (MCB-binding factor) (57). Both SBF and MBF are heterodimeric complexes sharing the same transactivation subunit, Swi6, and differing in their DNA-binding subunits: Swi4 for SBF and Mbp1 for MBF (3, 10, 47). The activity of these two transcription factors is essential for the initiation of the G_1/S transition, as demonstrated by the G_1-arrest displayed by *swi4 mbp1* and *swi4 swi6* cells (10, 47, 63). Optimal binding sites for Swi4 and Mbp1 have been determined. Swi4 recognizes the regulatory sequence 5'-CRCGAAA-3', called the Swi4/6-dependent cell cycle box or SCB (2, 10, 82). Mbp1 recognizes the regulatory sequence 5'-ACGCGN-3', called the *MluI* cell cycle box or MCB (47).

Genome-wide analyses globally mapping genomic binding sites for SBF and MBF have identified a broad range of potential gene targets for these transcription factors (36, 41, 77). Substantial overlap in the list of genes regulated by SBF and MBF was reported, suggesting partial redundancy in their functions (5, 41, 77). Though they show specificity for their respective consensus binding sites, it has been demonstrated that cross-recognition can occur, where SBF can bind to MCBs and MBF can bind to SCBs (47, 68, 84). This may offer an explanation for the overlap in gene targets seen for these two transcription factors. Another possibility for this overlap might be that some G_1/S-expressed genes (eg. *CLN2*) may contain both SCBs and MCBs in their promoters (80). This might suggest that a coordinated strategy may exist for the expression of these genes by SBF and MBF. Indeed, cells maintaining deletions of either *SWI4* or *MBP1* alone are viable, being able to initiate START processes such as budding and DNA replication (2, 10, 47). Therefore, some degree of functional redundancy may exist between these two transcription factors, and in fact, a recent study directly demonstrates that redundant positive control of SBF and MBF transcriptional activity is seen for a number of G_1/S-regulated genes (5). Nevertheless, both of these transcription factors do play somewhat specialized roles during the G_1/S transition, based on the classes of genes distinctly targeted by either regulator (41, 77). In general, SBF targets are predominantly involved in regulating bud-emergence, as well as membrane and cell wall biogenesis (39, 40). Also, other well characterized targets of SBF are the G_1-cyclins *CLN1* and *CLN2* (39, 80). MBF targets are mainly involved in regulating DNA replication and repair (5). Two well characterized targets of MBF are the S-phase cyclins *CLB5* and *CLB6* (47, 73).

Gene expression in eukaryotes is a complex process involving the activity of a number of different protein complexes and also employing dynamic structural changes of gene promoters (42). Important factors required for gene expression are chromatin-remodeling enzymes such as the Swi/Snf complex, and histone acetlytransferases (HATs) such as SAGA (Spt-Ada-Gcn5-Acetyltransferase) (11, 46, 65). The packaging of DNA by nucleosomes, creating an ordered chromatin structure, can deter the binding of transcription factors to regulatory sequences in gene promoters. Chromatin-remodeling enzymes can modify nucleosomes by altering nucleosome positioning and acetylating histone tails, thereby creating a more accessible chromatin structure and enhancing transcription. Sequence-specific transcription factors, like SBF and MBF, also play crucial roles in regulating transcription, specifically in promoting periodic gene expression. Sequence-specific transcription factors are important for the recruitment of many different factors to promoters, including the chromatin-remodeling enzymes, and also the general transcriptional machinery (42). The RNA-polymerase II holo-enzyme, which is required for the transcription of protein-encoding genes, is a large multisubunit complex. In yeast, the "core" RNA polymerase contains twelve subunits, Rpb1-12 (28). The Cdk7-equivalent kinase Kin28, the polymerase PolII, and the Srb/mediator complex are also components of the RNA-polymerase II holo-enzyme. Another dozen or more proteins comprising the general transcription factors TFIIA, TFIIB, TFIID, TFIIE, TFIIF and TFIIH, are other factors involved in transcription. Precise mechanisms employed by SBF and MBF in promoting transcription in yeast have not been fully characterized. However, the role of SBF in the transcriptional expression of the *HO* endonuclease gene in late-G_1 has been reported (17, 18). Transcription of the *HO* promoter involves an ordered recruitment of factors, and the SBF plays an important role in this recruitment process (18, 49). Recruitment of factors to the *HO* promoter begins during anaphase, with the expression of the Swi5 sequence-specific transcription factor. Swi5 binds to the *HO* promoter and then recruits the Swi/Snf nucleosome-remodeling complex, which itself recruits SAGA.

Chromatin remodeling by Swi/Snf and SAGA promotes the recruitment of SBF to *HO*. The binding of SBF to consensus SCBs in the *HO* promoter then permits the assembly of the pre-initiation complex, composed of the general transcription factors and the RNA-polymerase II holo-enzyme. Interestingly, the recruitment of RNA polymerase occurs in two steps: a Cln-Cdc28-independent recruitment (the SRB/mediator complex) and a Cln-Cdc28-dependent recruitment (the RNA-polymerase II core complex, Kin28 and the general transcription factors) (17). A similar mechanism has been proposed for the transcriptional activation of *CLN1* and *CLN2*. This two-step RNA polymerase II-recruitment wonderfully illuminates the close relationship between cell cycle regulation and gene expression. To date, similar studies investigating the mechanisms involved in MBF-mediated transcriptional activation have not been done, however, it is possible that a similar ordered recruitment is employed.

A number of structural studies comparing Swi4, Mbp1 and Swi6, have been able to assign functional regions within these proteins (30, 32, 56, 74, 84, 91). Though no DNA-binding domain is seen in Swi6, all three proteins comprising these two transcription factors share some common features. Intermolecular interactions between Swi6 and Swi4, or Swi6 and Mbp1 are thought to occur through homologous C-terminal heterodimerization domains (32). However, the most striking structural feature in this family of proteins is a conserved central region containing a series of sequence motifs called Ankyrin repeats (ANK domain) (30, 32, 74). ANK domains are believed to mostly mediate protein-protein interactions, however other possible functions have not been ruled out (58). One possible role for the ANK domains in SBF and MBF subunits, with respect to transcriptional activity, may involve interactions with cyclin-CDK complexes (76). ANK domains in both Swi4 and Mbp1 have been shown to bind to the B-type cyclin Clb2 *in vitro*. It has been proposed that the interaction between Clb2-Cdc28 and the SBF may lead to the inactivation of SBF transcriptional activity in G_2-phase (48). Whether a similar mechanism is involved in MBF inactivation has not been reported, however, regulation of these two transcription factors may differ considerably. Possible differences in SBF and MBF activity are evident from observations made of their respective gene targets. SBF appears to function as a transcriptional activator, promoting full expression and periodicity of its target genes (9, 10). Therefore, in *swi4* mutants, the expression of SBF-regulated genes (such as *HO*) is almost completely eliminated. In contrast, MBF appears to only impart a periodicity to its target genes (47). Analysis of MBF-regulated genes in *mbp1* mutants has revealed that these genes are expressed at a high constitutive level throughout the cell cycle. This constitutive expression maintains an intermediate level of RNA accumulation between the peaks and troughs observed for the periodic expression of these genes in proliferating wild type cells. This suggests that the MBF plays a dual role at its target genes, promoting expression during the G_1/S transition, and repressing expression during other stages of the cell cycle. Interestingly, multiple synthetic copies of the MCB consensus sequence are able to activate the transcription of a *lacZ* reporter gene, promoting the periodic G_1/S expression of this reporter in proliferating cells (54). Since MCBs on their own are able to initiate transcription of a *lacZ* reporter gene, and since Mbp1 appears to impart only a regulatory influence on this expression, the regulation of MBF-mediated genes may involve a complex mechanism employing other factors as transcriptional co-regulators of these genes.

When starved for nitrogen and fermentable carbon sources, diploid *S. cerevisiae* abandon mitotic growth and initiate a developmental program known as sporulation (59). Sporulation is a meiotic process leading to the production of haploid spores, a strategy devised by yeast for survival under challenging environmental conditions. Meiotic development involves one round of premeiotic DNA replication, followed by two meiotic divisions, meiosis I (MI) and meiosis II (MII), without an intervening S-phase. The orderly progression through sporulation is regulated by a cascade of gene expression, occurring in at least four distinct stages: early, middle, mid-late, and late (12, 59, 69). The induction of early meiosis-specific genes is dependent on the Ime1/Ume6 transcription factor complex (45, 79). Proteins encoded by early genes regulate early processes such as premeiotic DNA replication, chromosome pairing, synaptonemal complex formation, and meiotic recombination (12, 59). Early meiotic gene products also promote the expression of the meiosis-specific transcription factor Ndt80, a key regulator of middle gene expression (13, 66). Genes targeted by Ndt80 are required for the meiotic nuclear divisions and spore formation (12, 13). Though their roles in promoting the mitotic G_1/S transition have been fairly well established, little is known about whether SBF and MBF function in sporulation. It has been shown that many genes predominantly regulated by the SBF, such as the G_1-cyclins *CLN1* and *CLN2* are not expressed in sporulating cells (15, 70). Also, cells lacking Swi4 or Swi6 efficiently proceed through sporulation (25, 52), further suggesting that the SBF is not required for meiotic development. The absence of SBF activity in sporulation seems appropriate since processes such as budding should not occur. However, DNA replication is required during meiotic development, and it has been demonstrated that many MBF-regulated genes are expressed during sporulation (41).

Work presented in this chapter demonstrates that Mbp1 is constitutively expressed during meiotic development and this transcription factor does regulate a subset of MCB-containing genes during early stages in sporulation. Interestingly, in striking contrast to mitotic growth, Mbp1 does not influence the crucial meiotic expression of *CLB5*. Also, Mbp1 is not essential for premeiotic S-phase, meiotic recombination or efficient spore formation. Our work demonstrates that MBF is functional during sporulation however its transcriptional activity may be regulated differently compared to mitotic growth. Also, our analysis suggests a meiosis-specific regulation for the S-phase cyclin *CLB5* and may indicate the existence of another transcription factor regulating premeiotic DNA replication in sporulating cells.

III.2 – Results and Discussion

III.2.a. – Transcriptional profile of the S-phase cyclin *CLB5* during meiotic development: *CLB5* encodes a B-type cyclin, and is periodically expressed at the G_1/S transition during mitotic proliferation (29, 51, 73). Clb5, along with the coordinately expressed cyclin Clb6, activate Cdc28 during mitotic S-phase and play an important role in activating DNA replication (27, 29, 73). The periodic expression of *CLB5* during mitosis has been attributed to regulation by the START-specific transcription factor MBF (47, 73). It has been demonstrated that periodicity of *CLB5* transcription is not seen in *mbp1* mutants synchronized during growth (47). Also, periodic expression of *CLB5* is lost in *swi6* mutants, but is not affected in *swi4* strains (73). During sporulation, *CLB5* and *CLB6* expression is essential for the initiation of premeiotic DNA replication (81). In cells undergoing meiotic development, *CLB5* transcripts accumulate in a temporal pattern that is surprisingly distinct from that seen in mitotic cells. Meiotic *CLB5* transcription begins early, with mRNA appearing within 2 hours of meiotic induction (Figure III-1, Wild Type). This expression coincides with the onset of premeiotic DNA replication (Figure III-3A, see analysis of DNA content in wild type cells). Peak transcript accumulation, however, is seen between 5 to 8 hours into sporulation, concurrent with the onset of middle sporulation events such as the meiotic nuclear divisions (MI and MII) (Figure III-1, Wild Type). This is in striking contrast to the G_1/S peak observed for mitotically expressed *CLB5* (47). Transcriptional induction of *CLB5* during sporulation is dependent on the initiation of the meiotic program, since *CLB5* does not accumulate in *ime1* mutants, which are unable to induce the expression of meiosis-specific genes (Figure III-1, Δ*ime1*). However, in *ime2* mutants, in which early gene expression does occur, *CLB5* mRNA expression is induced and accumulates significantly (Figure III-1, Δ*ime2*).

Very little is known about the regulation of *CLB5* expression during meiotic development. Even though many MCB-containing genes are expressed during sporulation, and many of the same DNA replication genes functioning in growth are also required during premeiotic S-phase (41), a role for the MBF in sporulating cells has not been previously reported. In striking contrast to its reported role in mitotic proliferation, we find that MBF does not seem to regulate *CLB5* transcription during sporulation. No difference in the *CLB5* mRNA profile was observed between wild type diploids and *mbp1* mutants (Figure III-1, compare Wild Type to Δ*mbp1*). Even though SBF has demonstrated MCB-binding activity, it does not influence *CLB5* expression during growth (29, 73), and so it is not surprising that inactivation of *SWI4* does not reduce *CLB5* expression during sporulation either (Figure III-1, compare Wild Type to Δ*swi4*). We did observe that the peak accumulation of *CLB5* seen during middle sporulation stages appears slightly earlier in the *swi4* strain than in wild type cells. This early accumulation may be due to an earlier induction of meiotic development that might be occurring in *swi4* mutants. The expression of *CLN1* and *CLN2*, well established SBF targets, is severely reduced in *swi4* strains (22, 63, 64, 80). Since Cln activity has been shown to repress *IME1* expression during proliferation (15, 70), a reduction in the *CLN* expression during growth may promote a more efficient induction of the meiotic program upon transfer to sporulation conditions. This may result in a perceived shortening of early meiotic processes in our assay.

The surprising observation that Mbp1 does not affect *CLB5* expression during meiotic development encouraged a further analysis of Mbp1 in sporulating cells. *MBP1* mRNA is constitutively expressed throughout the meiotic time course (Figure III-2A, *MBP1*), and we find this is consistent with the uniform Mbp1 protein abundance clearly detectable throughout sporulation (Figure III-2B, Mbp1-Myc). A previous study has reported that Swi6 is also present during meiotic development (14), suggesting that Swi6 and Mbp1 may be associating into the MBF complex during sporulation as well. In contrast to the constitutive abundance of Mbp1 detected, and the reported expression of Swi6, we observed an apparent instability of Swi4 protein in sporulating cells (Figure III-2C, Swi4-HA). Full length Swi4-HA, seen in proliferating cells (Figure III-2C, YEPD lane), was not detected in cells undergoing meiotic development. Instead, only lower molecular weight degradation products were observed in these cells (Figure III-2C), implicating that an immediate inactivation of the SBF occurs upon meiotic induction. This is consistent with previous reports demonstrating that SBF-mediated genes, such as *CLN1* and *CLN2*, are not expressed during meiotic development (15, 70). The difference in the stability of these two transcription factors, MBF and SBF, may serve as a key regulatory mechanism allowing the activation of MCB-driven genes during sporulation, while preventing the expression of SBF targets. Such partitioning of SBF- and MBF-regulated genes is definitely critical for meiotic development, and this provides an explanation for why two related transcription factors have evolved in budding yeast to coordinate seemingly coincident events at START in proliferating cells.

The peak accumulation of *CLB5* occurring between 5 to 8 hours, coincident with the middle stages of sporulation, has been reported previously (13). Middle sporulation expression of the *CLB* genes, including *CLB5*, is dependent on the meiosis-specific transcription factor Ndt80. We confirm that deletion of *NDT80* abolishes the peak accumulation of *CLB5* during middle sporulation (Figure III-1, Δ*ndt80*). However, *CLB5* is still expressed at a constitutive level in these cells indicating another factor is promoting this transcription. Even though we detected no alteration of meiotic *CLB5* expression in *mbp1* mutants, we considered the possibility that any Mbp1 influences on this expression might have been masked by the strong Ndt80-induced transcription. However, deletion of both *MBP1* and *NDT80* produced no added defects to *CLB5* expression, resulting in an accumulation and abundance similar to that seen in *ndt80* mutants alone (Figure III-1, compare Δ*ndt80* Δ*mbp1* to Δ*ndt80*). Together, these observations demonstrate that, even though Mbp1 is present, this transcription factor does not play an essential role in *CLB5* mRNA expression during meiotic development. Also, this data suggests that another unknown factor induces the expression of *CLB5* during sporulation.

III.2.b. – Mbp1 is not required for the timely induction of premeiotic DNA replication and efficient meiotic recombination: Even though Mbp1 does not appear to regulate the essential meiotic expression of the S-phase cyclin *CLB5*, Mbp1 may still be influencing other genes during sporulation and may still have a role in regulating premeiotic S-phase. Wild type SK1 diploids initiate premeiotic DNA replication around 2 hours post induction and complete this process at approximately 4 hours into the sporulation program (Figure III-3A, see flow cytometry analysis of *MBP1 NDT80* wild type cells). Analysis of the DNA content for *mbp1* mutants throughout sporulation revealed no defects in the timing of meiotic S-phase compared to wild type cells (Figure III-3A, *mbp1 NDT80*). Also, no defects were seen in *ndt80* cells, which should have no trouble initiating premeiotic S-phase (Figure III-3A, *MBP1 ndt80*). Interestingly, this analysis indicates that Mbp1 is not critical for the successful initiation and completion of premeiotic DNA replication.

Extensive homologous recombination during meiotic development is a hallmark of most sexually reproducing eukaryotes (4, 50). In *S. cerevisiae*, efficient meiotic recombination is critically dependent on the successful completion of DNA replication (6, 78). This link between premeiotic DNA replication and meiotic recombination can be used to analyze the efficiency of

DNA synthesis, since any delays in the progression of meiotic S-phase would manifest as a delay in the accumulation of recombination events. Wild type and *mbp1* mutants were compared in a "return to growth" assay, analyzing intragenic meiotic recombination at two separate loci, *ARG4* and *HIS4* (Figure III-3B). Both diploid strains, heteroallelic for these two loci, were induced to sporulate and samples of cells were collected every two hours. Collected samples were appropriately diluted and spread onto YEPD (~500 cells/plate) and selection plates (~1×10^6 cells/plate, -arg and –his agar), and these plates were then assayed for colony growth. Essentially, reacquisition of the Arg+ or His+ phenotypes would serve as a marker for a meiotic recombination event, and an accumulation of this phenotype would indicate a relative frequency of recombination occurring in a given population of cells. We observed that the rate at which recombinants were recovered between *MBP1* wild type and *mbp1* mutant sporulating cells was very similar, with recombinants accumulating in a timely manner and to levels comparable to each other (Figure III-3B). Together, the data presented in Figure III-3 clearly demonstrate that deletion of *MBP1* does not produce any defects in premeiotic DNA replication or in meiotic recombination. This suggests that the MBF may not play an important role during meiotic development. Our recombination results may actually conflict with a study by Leem et al. (1998) investigating the meiotic role of Swi6. In this study, reduced spore viability was observed in sporulating *swi6* mutants (52). Microscopic examination of the dissected spores revealed problems in germination, which led to the proposal that *swi6* mutants display defects in meiotic progression. In agreement with our *mbp1* data, sporulating *swi6* cells were able to efficiently progress through premeiotic DNA replication. However, these mutants demonstrated a significantly reduced frequency of recombination. Leem et al. (1998) also reported that, similar to *swi6* mutants, significantly reduced spore viability was seen in sporulating *swi4* cells as well (52). Since we have observed Swi4 to be immediately degraded upon meiotic induction (Figure III-2C), and since SBF-regulated genes are not expressed during sporulation (15, 70), we have to contend that the reduced spore viability displayed by *swi4* mutants are most probably not be due to defects incurred during meiotic progression. During mitotic proliferation, the SBF is involved in diverse cellular processes. Along with regulating the expression of the G_1 cyclins *CLN1* and *CLN2* (39, 80), SBF also plays important roles in the maintenance of cell integrity and cytokinesis (8, 39, 40). Therefore, it is reasonable to propose that cell integrity defects in *swi4* mutants may compromise cells undergoing meiotic development, during germination, and during the initial rounds of mitotic growth after germination of a spore. This could explain the reduced spore viability observed in these cells. It is important to note that Swi6 is also a component of the SBF, which may complicate analysis of the data demonstrating the potential role for Swi6 during meiotic development (52). It is quite possible that the reduced recombination frequency seen in *swi6* mutants, which was monitored using a similar "return to growth" assay, may actually be the result of cell integrity defects, and may not be due to defects in meiotic recombination.

III.2.c. – Mbp1 regulates a subset of MCB-mediated genes during meiotic development: Since deletion of *MBP1* does not seem to affect premeiotic DNA replication or meiotic recombination we acknowledged the possibility that Mbp1, and by extension the MBF, may not be regulating gene expression during sporulation. Even though both Mbp1 and Swi6 are present, they may not be able to form a complex, or the MBF may be kept in an inactive state by some unknown mechanism. To properly address this possibility, we analyzed the expression of other MBF-regulated genes in sporulating *mbp1* mutants. Two important DNA replication genes, *RNR1* (which encodes the large subunit of Ribonucleotide Reductase) and *TMP1* (which encodes Thymidilate Synthase), contain MCB elements in their promoters, and are well established MBF-regulated targets (5, 26, 41, 47, 53, 77, 88). Both *RNR1* and *TMP1* demonstrate a characteristic pattern of mRNA expression in sporulating wild type cells, displaying a distinct peak of transcript accumulation occurring between 2 and 6 hours post meiotic induction (Figure III-4A, *RNR1* and *TMP1*). This peak accumulation appears coincident with the onset of premeiotic S-phase, and therefore may be seen as a G_1/meiotic S-phase expression. Surprisingly, the periodic expression of *RNR1* and *TMP1* were abolished in *mbp1* mutants, instead being replaced by a relatively uniform level of expression (Figure III-4B, *RNR1* and *TMP1*). Therefore, we find that both *RNR1* and *TMP1* are, in fact, meiotic targets of Mbp1 activity, and therefore demonstrate an MBF-dependent expression. No defect in meiotic *CLB5* expression was again seen in the *mbp1* mutants (Figure III-4B, *CLB5*), and the timely expression of *SPS1* and *SPS2*, two characteristic middle sporulation genes, distinctly demonstrates that the progression of sporulation was not affected (Figure III-4B, *SPS1,2*). We also note the differing meiotic expression profiles between *CLB5* (MBF-independent), and *RNR1* and *TMP1* (MBF-dependent). Peak expression of *RNR1* and *TMP1* at G_1/meiotic S-phase clearly distinguishes these two MBF-regulated genes from *CLB5*, and illuminates an MBF-driven transcriptional profile during meiotic development.

III.2.d. – A differential role for MBF during meiotic development: The intriguing observation that Mbp1 regulates *RNR1* and *TMP1* but not *CLB5*, suggests an interesting new possibility regarding MBF regulation that has not been previously considered. It appears that Mbp1, and hence the MBF, might be regulating only a subset of MCB-containing genes during meiotic development. Indeed, the differential regulation of three established mitotic MBF targets during sporulation (Figure III-4A and -4B, *CLB5*, *RNR1*, and *TMP1*) strongly supports this proposition. The nature of the meiosis-specific mechanisms functioning to mediate MBF regulation during sporulation remains completely up to conjecture, however evidence exists to offer some possible explanations.

Chromatin remodeling plays in important role in the regulation of gene expression during sporulation in budding yeast. Repression of early meiotic genes during growth by chromatin modifying enzymes has been fairly well characterized (35, 43, 44, 86). The repression of Ime1-regulated early genes during mitotic proliferation has been clearly shown to depend on a complex comprising the DNA-binding protein Ume6 and the Sin3-Rpd3 histone deacetylase (HDAC) complex (43, 44). Repression of early genes has also been shown to require the ISWI class of ATP-dependent chromatin-remodeling factors (35, 86). ATP-dependent chromatin-remodeling complexes such as ISWI (and also Swi/Snf) use the energy of ATP hydrolysis to modify nucleosome positioning along a DNA template, or alter nucleosome composition (62). HDACs deacetylate histone tails, thereby altering histone-DNA contacts. Together, these factors work to produce a chromatin structure inaccessible to potential transcriptional regulators. In budding yeast, *isw2* mutants exhibit defects during early sporulation (86). The Isw2 complex has been observed to work in a parallel pathway to the Sin3-Rpd3 complex, and it appears to be recruited to target genes by Ume6 as well (31, 35). The modification of chromatin structure at meiotic genes may suggest that a change in chromatin dynamics occurs throughout the genome during sporulation. Such differential regulation of chromatin between growth and meiotic development could explain the differential activity of MBF during sporulation. Perhaps meiosis-specific variations of promoters such as *CLB5* might alter MBF specificity for these targets genes.

Along with possible differences in chromatin structure for intended targets, meiosis-specific regulation of the MBF may also occur, remodulating its activity during sporulation. Both Swi4 and Swi6 have been shown to be phosphorylated in proliferating cells (1, 33, 75, 82, 87). In particular, Swi6 has been shown to be phosphorylated in a CDK-dependent manner, which regulates its nuclear localization (33, 75). To our knowledge, Mbp1 has not been shown to be phosphorylated. However, a reasonable mechanism to explain the distinct function of Mbp1 during sporulation could involve meiosis-specific modifications of this transcription factor. Such modifications may promote interactions with other factors already bound to certain MCB-containing promoters, or alter Mbp1's sequence binding determinants.

Recently, the SBF-associated protein Whi5 has emerged as a transcriptional repressor of SBF activity, and mediates the Cln-dependent activation of this transcription factor (19, 24). Though no such repressor has been found to regulate MBF, other associated factors may be influencing MBF activity during mitotic proliferation and meiotic development. In fact, a number of proteins have been shown to associate with Swi6 and Mbp1, and these proteins may act as potential regulators of MBF-mediated transcription. *SKN7* was discovered in a screen looking for additional activators of MCB- and SCB-dependent transcription (61). Overexpression of *SKN7* bypasses the essential requirement for both SBF and MBF during growth. The Skn7 protein can bind to Mbp1, and in the absence of *SWI6*, this protein becomes necessary for Mbp1-mediated transcription (7). Interestingly, this implies that Mbp1 and Skn7 can form a transcription factor independent of MBF. However, no role for Skn7 during sporulation has been reported. Another protein, Stb1, has been shown to associate with Swi6 and localize to the promoters of MBF-regulated genes (20, 37). Stb1 is believed to be a regulator of MBF activity, since deletion of *STB1* requires *SWI4* for viability, and cells lacking *STB1* were specifically defective in expressing an MCB::*lacZ* reporter gene (20). Though Stb1 appears to influence MBF transcriptional activity, the nature of this regulation has not been fully characterized.

An intriguing feature of the MBF in *Schizosaccharomyces pombe* (also called the DSC1 complex) may offer some clues to the unique MBF activity observed during meiotic development in *S. cerevisiae*. Two distinct variants of DSC1 are apparent in fission yeast. Both contain the Swi6 homologue Cdc10 (10, 55). Cdc10 binds to either Res1 or Res2, both members of Swi4/Mbp1 protein family, and both demonstrating specificity towards MCB sequences (60, 83, 91, 92). Interestingly, it has been demonstrated that the Res1-Cdc10 DSC1 complex is the most important regulator of START-specific transcription during mitotic growth, whereas the Res2-Cdc10 DSC1 complex is the primary regulator of premeiotic DNA replication and meiotic recombination (60, 83, 92). Recently, two other proteins have also been shown to be co-regulators with DSC1 in *S. pombe*, and are required for proper transcriptional activity of this complex. These are the proteins Rep1 and Rep2 (23, 90). An intriguing observation about these co-regulators is that *rep1*$^+$ is exclusively expressed during meiotic development, and *rep2*$^+$ is only expressed during mitotic proliferation (23). Not only does this support the possible existence of meiosis-specific co-regulators for MBF, but it also implicates the possibility of meiosis-specific variants of this transcription factor in *S. cerevisiae*.

Our observation that Mbp1 does not influence *CLB5* expression during sporulation has clearly implicated another factor regulating this MCB-regulated gene during meiotic development. An evaluation of *CLB5* expression during growth has also provided evidence to suggest the existence of novel factors that may regulate MCB-containing genes in budding yeast (47). Though *CLB5* expression has proven to be dependent on Mbp1 in cultures synchronized by centrifugal elutriation, when cells are synchronized by deprivation then re-induction of G_1-cyclin activity, *CLB5* mRNA displays a similar periodic expression in both wild type and *mbp1* cell. This is in contrast to *TMP1* and *POL1*, which both demonstrate a loss of periodicity in *mbp1* cells synchronized by either protocol. This data suggests that high Cln-Cdc28 activity may be able to promote periodic *CLB5* expression independently of Mbp1, and implicates an alternate regulation for this gene versus other MCB-mediated targets. Other than cross-recognition by Swi4, no other MCB-binding proteins have been uncovered in budding yeast. However, *S. cerevisiae* encodes several candidate transcription factors (*SOK2, PHD1* and *GAT1*) that maintain some sequence similarity to the DNA-binding domain of Mbp1 (91). *GAT1* encodes a GATA-family transcription factor involved in activating genes required for the catabolism of poor nitrogen sources (16, 21, 38, 85). *SOK2* and *PHD1* are developmental regulators of pseudohyphal growth in budding yeast (67, 71, 72, 89). When exposed to environmental conditions of limited available nitrogen, diploid *S. cerevisiae* cease normal vegetative growth and may begin growing into pseudohyphae (34).

Pseudohyphal development allows budding yeast to form branched chains of connected cells that are capable of invading their growth substrate. *SOK2* and *PHD1* respond to the signals initiating this developmental pathway (67, 71, 72, 89). Therefore, Sok2, Phd1 and Gat1 are regulators that respond to a poor nitrogen environment, a condition also influencing the induction of sporulation. Whether these transcription factors are involved in meiotic development has not been determined.

III.2.e. – Conclusion: In this study, we demonstrate a role for the START-specific transcription factor Mbp1 during sporulation. Mbp1 is expressed during meiotic development, and activates a subset of MCB-containing genes, such as *RNR1* and *TMP1*. Deletion of *MBP1* results in a constitutive level of expression for its targets during sporulation, consistent with results seen during mitotic growth in *mbp1* mutants (47). Though inactivation of *MBP1* does not produce any defects in premeiotic DNA replication or meiotic recombination, it is possible that any subtle effects may be masked by laboratory conditions. More challenging environments in nature may require a more robust expression of DNA replication genes during premeiotic S-phase, making Mbp1 activity more important for this process. Finally, we propose the existence of unique regulatory mechanisms controlling the expression of *CLB5* during sporulation. Meiotic *CLB5* transcription remains independent of Mbp1 activity during early sporulation, and may be regulated by unique meiosis-specific factors. Further evaluation of *CLB5* transcriptional regulation during meiotic development is warranted (see Chapter IV).

III.3 – References

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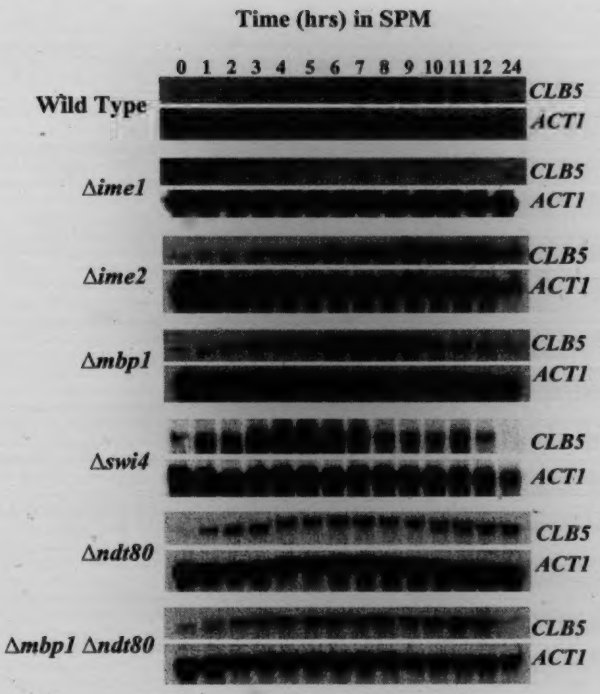
Time (hrs) in SPM
0 1 2 3 4 5 6 7 8 9 10 11 12 24
Wild Type
CLB5
ACT1
Δime1
CLB5
ACT1
Δime2
CLB5
ACT1
Δmbp1
CLB5
ACT1
Δswi4
CLB5
ACT1
Δndt80
CLB5
ACT1
Δmbp1 Δndt80
CLB5
ACT1

Figure III-1: *CLB5* expression during sporulation is independent of *MBP1*. Homozygous diploid cells, either Wild Type or harboring the deletion(s) indicated to the left were induced to sporulate. Samples collected at indicated time-points were processed for the extraction of RNA. Samples were then subjected to Northern blot analysis. ^{32}P-labeled probing for *CLB5* mRNA reveals the transcriptional profile for this cyclin in each strain undergoing meiosis. ^{32}P-labeled probing for *ACT1* transcripts serves as a loading control.

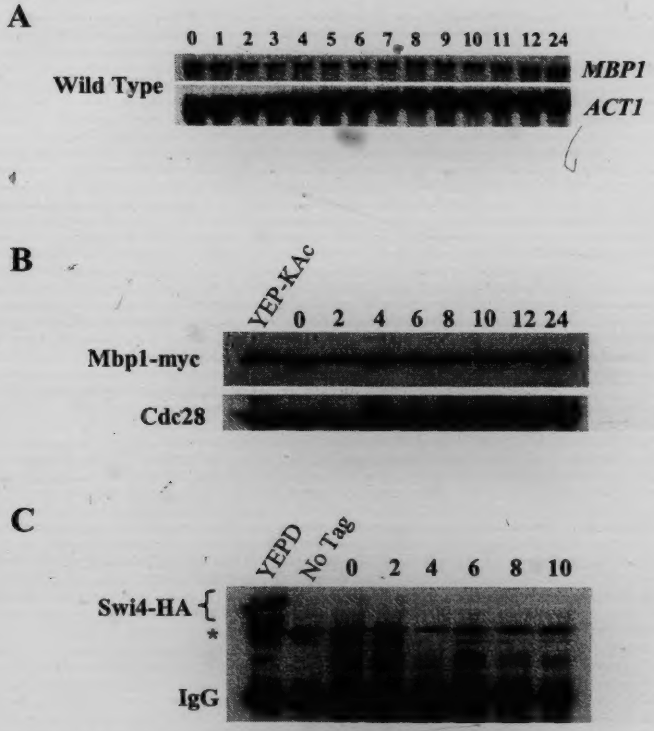

Figure III-2: Mbp1 is constitutively abundant throughout meiotic development, but Swi4 protein is unstable in sporulating cells. (A) Diploid Wild Type cells were induced to sporulate and samples were collected at indicated time-points for Northern blot analysis. *MBP1* and *ACT1* transcript profiles were visualized by ^{32}P-labeled probing. (B) A homozygous diploid strain containing tagged alleles of Mbp1 was induced to sporulate, and samples were prepared for Western blot analysis. Mbp1-Myc was visualized with anti-Myc antibodies. Immunodetection of Cdc28 (anti-PSTAIRE) for the same samples was done as a loading control. (C) Equal samples from a homozygous diploid Swi4-HA strain undergoing meiotic development were collected at the indicated time-points. Extracts made were subjected to immunoprecipitation with anti-HA antibodies. The immunocomplexes were probed by Western blot for the HA epitope. Cross-reacting IgG is indicated. Also a non-specific background HA-reactive species was detected, indicated by an asterisk (*).

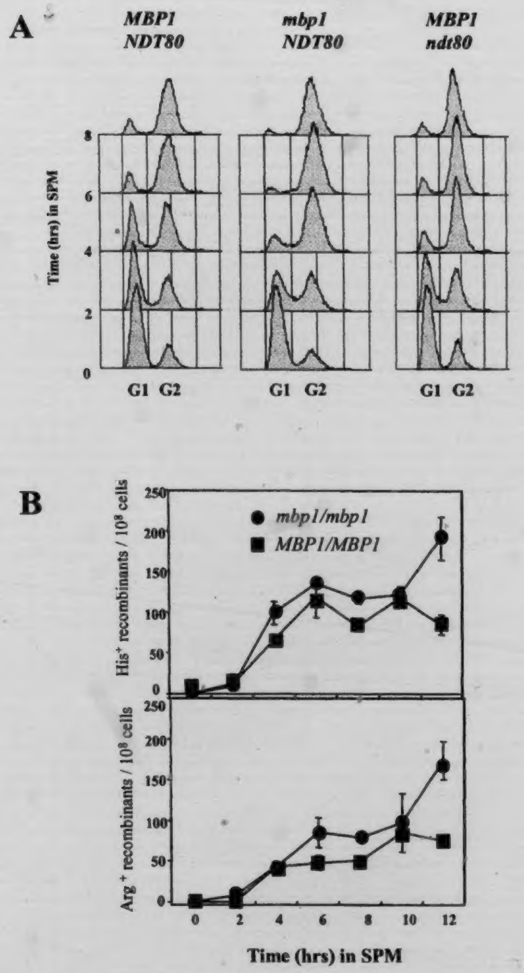

Figure III-3: *MBP1* is not essential for effective completion of premeiotic DNA replication or meiotic homologous recombination. (A) Samples collected from homozygous *MBP1 NDT80* (wild type), *mbp1 NDT80*, and *MBP1 ndt80* diploids undergoing meiotic development were analyzed for DNA content by flow cytometry. G1 (2C) and G2 (4C) peaks are indicated beneath the overlay plots. (B) Homozygous meiotic recombination in *mbp1* (●) or *MBP1* (■) diploids, heteroallelic for *HIS4* and *ARG4*, was measured by the appearance of His+ or Arg+ recombinants in samples taken from sporulating cultures at the indicated time-points.

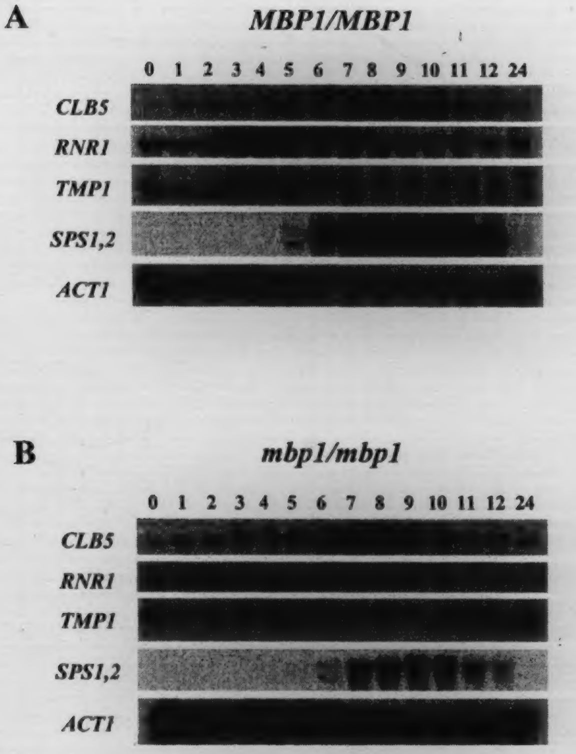
A
MBP1/MBP1
0 1 2 3 4 5 6 7 8 9 10 11 12 24
CLB5
RNR1
TMP1
SPS1,2
ACT1
B
mbp1/mbp1
0 1 2 3 4 5 6 7 8 9 10 11 12 24
CLB5
RNR1
TMP1
SPS1,2
ACT1

Figure III-4: *MBP1* regulates a subset of MCB-containing genes during meiotic development. *MBP1* wild type (A) and *mbp1* mutant (B) homozygous diploids were induced to sporulate and samples were collected for RNA extraction at time-points indicated. Samples were subjected to Northern blot analysis for transcripts shown at left. *SPS1,2* expression represents the relative onset of middle sporulation stages. *ACT1* was probed for a loading control.

Chapter IV

Meiosis-Specific Regulation of the S-phase Cyclin *CLB5* Requires *MluI* Cell Cycle Box (MCB) Elements in Its Promoter but is Independent of MBF Activity

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IV.1 – Introduction

DNA replication in eukaryotic cells is a highly intricate process involving the coordinated regulation of a large number of protein complexes, ensuring the timely initiation of S-phase, and the faithful duplication of chromosomes (1). A critical event in the initiation of DNA synthesis is the activation of cyclin-dependent kinase activity. In the budding yeast, *Saccharomyces cerevisiae*, the B-type cyclins Clb5 and Clb6 are the principle S-phase promoting cyclins, activating Cdc28 at the G_1/S transition (11, 12, 20, 32). Clb5 appears to play a more dominant role in promoting DNA replication. In proliferating cells, mutants deficient for *CLB5* are able to initiate DNA replication in a timely fashion, however, S-phase lasts twice as long (12, 20, 32). *clb5* mutants appear unable to activate late replication origins, resulting in reduced origin usage and a lengthened S-phase (11). Inactivation of *CLB6* does not appear to negatively influence the onset or duration of mitotic S-phase (20, 32), and *clb6* mutants show no noticeable defects in firing of replication origins (11). Results from these single mutant data have suggested that Clb5-Cdc28 can activate both early and late origins, while Clb6-Cdc28 can only fire early origins (11). However, other data arguing against any inherent origin specificity for Clb5 and Clb6 has recently emerged. Clb6 lacks the destruction-box motif which is targeted by the Anaphase Promoting Complex (APC) (16). Instead, this cyclin contains potential Cdc4 degron motifs and may be a target of the SCF^{Cdc4} ubiquitin ligase (16). Clb6 is degraded rapidly at the G_1/S boundary, consistent with it being a target of the SCF^{Cdc4}, while Clb5 persists until anaphase. This suggests that the limitation of Clb6 in a *clb5* mutant may have more to do with decreased S-phase cyclin-CDK activity than functional differences in cyclin specificity. Indeed, increased Clb6 dosage (13) or hyperstabilized Clb6 expressed from its own promoter (16) rescues the extended S-phase defect seen in *clb5* mutants. Inactivation of both *CLB5* and *CLB6* produces a noticeable delay in the initiation of DNA replication, however, the actual duration of S-phase appears similar to that of wild type cells (11, 20, 32) Functional redundancy attributed to Clb1-4 activity has been shown to compensate for the loss of *CLB5* and *CLB6* in these double mutant cells, since mutants deficient for all six *CLB*s are completely unable to initiate DNA replication (31).

Under conditions of environmental stress related to nutrient deprivation, diploid budding yeast cells are able to abandon mitotic proliferation and enter a developmental program called sporulation (24). This specialized process involves the progression through a meiotic-cell cycle and the formation of haploid spores. Meiosis is characterized by one round of premeiotic DNA replication, followed by two nuclear divisions (MI and MII) without an intervening S-phase. It has been determined that many of the same genes and machinery required for mitotic DNA replication are also required during premeiotic S-phase (21, 25, 27, 33, 35, 39). In fact, DNA replication during meiotic S-phase appears to initiate from the same origins as mitotic DNA replication (9). However, unique characteristics of premeiotic S-phase such as recombination, suggest that DNA replication during sporulation may employ different regulatory mechanisms. An interesting feature exemplifying this unique meiotic regulation of DNA synthesis is the distinct requirement for *CLB5* and *CLB6* during premeiotic S-phase. As in growth, *CLB5* and *CLB6* regulate premeiotic DNA replication, however, these cyclins are absolutely essential for this process (34). Sporulating *clb5 clb6* double mutants fail to initiate DNA replication altogether, even though the expression of *CLB1-4* is not dramatically altered in these cells. Apparently, the functional redundancy seen in Clb1-4 during mitotic growth does not apply to the regulation of premeiotic DNA synthesis. This highlights a significant difference between meiotic S-phase and mitotic S-phase.

A number of different possibilities exist to explain the essential requirement for *CLB5* and *CLB6* in sporulation. The specificity of cyclins towards various targets has been proposed as a mechanism promoting specialization of cyclin-CDK activity. It has been reported in proliferating cells that Clb5-Cdc28 activity appears to show specificity towards a proportion of Cdc28 targets, being able to more efficiently modify these targets compared to Clb2-Cdc28 (22). This Clb5 specificity is dependent upon a targeting domain found on the surface of this cyclin, called the hydrophobic patch motif (HPM), which binds to a short sequence in its substrates called the RXL or Cy motif. *CLB2* placed under the regulation of the *CLB5* promoter is unable to fulfill *CLB5*'s role with respect to efficient phosphorylation of these Clb5-specific targets (22), and also fails to rescue the S-phase defects seen in *clb5 clb6* mutants (10). These results imply that Clb5 and Clb6 demonstrate some degree of specificity towards their S-phase targets that may not be employed by Clb1-4. However, in contradiction to the proposed intrinsic specificity of cyclin activity, it has been recently demonstrated that inactivation of the CDK inhibitory kinase *SWE1* (*Saccharomyces* Wee1), in combination with early *CLB1-4* expression, allows these mitotic cyclins to initiate DNA replication with similar effectiveness to Clb5 (15). Swe1 may specifically inhibit Clb1-4 without affecting Clb5 and Clb6, since over-expression of *SWE1* inhibits mitotic functions attributed to Clb1-4, however DNA replication remains unaffected (2). This data would suggest that, rather than an intrinsic cyclin specificity, extrinsic factors such as modification of activity and temporal expression may be responsible for the apparently specialized cyclin activity observed. Differential temporal expression may, in fact, be a major factor determining the essential nature of *CLB5* and *CLB6* during premeiotic DNA replication. The failure of Clb1, Clb3 and Clb4 to rescue the *clb5 clb6* replication defect may be due to their later accumulation during sporulation. *CLB1-4* expression rapidly follows that of *CLB5* and *CLB6* during proliferation, which may accommodate the functional redundancy seen among the *CLB*s in growth. However, due to the extended length of premeiotic S-phase, a greater amount of time passes between the initiation of DNA replication and the expression of *CLB1*, *CLB3* and *CLB4* in sporulation. These Clbs may not accumulate in time, possibly precluding them from complementing a *clb5 clb6* meiotic defect. This later expression, possibly hindering any functional redundancy among the *CLB*s, has led to the proposal of a window of opportunity for S-phase CDK activity during meiotic development (34). A reasonable basis for this window of opportunity could be that potential instabilities may be inherent in the pre-replication complex (pre-RC) (34). Activation of DNA replication may require the activity of Clb-Cdc28 prior to the degradation of the labile pre-RC state.

Meiotic development involves a dramatic reprogramming of cell cycle regulation to accommodate unique aspects of this specialized progression. Many meiosis-specific transcription factors are employed to coordinate this reprogramming, and it is reasonable to propose that those genes expressed in both sporulation and growth may fall under different regulatory controls during each process. With respect to *CLB5*, unique transcriptional regulation may be a strong determinant further enforcing the specialized activity of this S-phase cyclin during premeiotic DNA replication. In proliferating cells, *CLB5* expression maintains a distinctive G_1/S peak, and has been shown to be regulated by the MBF (MCB-Binding Factor) (19). The MBF is a heterodimeric transcription factor composed of the DNA-binding subunit Mbp1 and the trans-activation subunit Swi6 (19). The MBF binds to specific consensus elements in the promoters of its target genes known as MCBs (*MluI* cell cycle boxes; 5'-ACGCGN-3'). A number of MCB sequences have been identified in the *CLB5* promoter, and MBF has been shown to associate with this region (12, 32). However, we have demonstrated that the MBF does not influence *CLB5* expression during meiotic development (see

Chapter III). Also, *CLB5* does not display a G_1/meiotic S-phase peak seen for other MBF-regulated genes during sporulation (eg. *RNR1* and *TMP1*). These findings strongly suggest that *CLB5* expression during sporulation may be mediated by another factor which may be part of a meiosis-specific mechanism. Such a mechanism may include strategies to exclude MBF influences, and may involve meiosis-specific regulators inducing the early-meiotic expression of *CLB5*. In this chapter we investigate the transcriptional regulation of *CLB5* through an in-depth analysis of the *CLB5* promoter. Specifically, we focus on regulatory sequences affecting premeiotic DNA replication. Though we have confidently established the lack of MBF influence on meiotic expression of *CLB5*, we surprisingly found that transcription of this cyclin is still dependent on MCB sequences in its promoter. Inactivation of these MCBs dramatically alters *CLB5* expression during sporulation and also produces defects in premeiotic DNA replication. Our findings highlight the importance of transcriptional regulation on *CLB5* activity, and implicate a novel meiosis-specific MCB-binding factor influencing *CLB5* expression.

IV.2 – Results and Discussion

IV.2.a. – The S-phase cyclin *CLB5* is essential for efficient sporulation: The SK1 background is known for its efficiency in sporulation, with wild type diploids approaching a sporulation frequency of approximately 90%, and displaying almost 100% spore viability (Table IV-1). *clb5 clb6* mutants, however, are unable to form complete asci 24 hours after the induction of meiotic development, and show zero spore viability (Table IV-1). Though this defect in *clb5 clb6* cells is most likely the result of an inability to initiate S-phase (34), it could be possible that potential defects in chromosome metabolism persist into meiotic development, preventing successful DNA replication. To address this, we generated a strain expressing *CLB5* solely from an *IME2* promoter, limiting *CLB5* transcription to sporulation. Proliferating *clb5 clb6 IME2-CLB5* diploids displayed a similar S-phase defect to that of the parental *clb5 clb6* double mutants. During sporulation, *clb5 clb6 IME2-CLB5* cells progressed through meiotic development at a similar rate as *CLB5 CLB6* cells, achieving a high degree of tetrad formation (83%) and spore viability (96%) (Table IV-1). This indicates that *de novo* expression of *CLB5* during sporulation is sufficient to support effective sporulation. Another strain was created expressing *CLB5* from a methionine-repressible promoter (*clb5 clb6 MET3-CLB5*). When grown in rich medium supplemented with methionine (*MET3* promoter off), then transferred to sporulation medium lacking methionine (*MET3* promoter on), *clb5 clb6 MET3-CLB5* cells effectively completed MII (72%), achieving a sporulation efficiency of 67% (Table IV-1). A noticeable decrease in tetrad formation was detected in these cells, however spore viability was similar to that seen in wild type cells (97%). Under alternate conditions, when these cells were grown in the absence of methionine (*MET3* promoter on), then induced to sporulate in the presence of methionine (*MET3* promoter off), the efficiency of tetrad formation was significantly reduced, with only 24% of the cells completing two meiotic divisions and only 10% forming complete asci (Table IV-1). 1mM of methionine was used in this experiment, which caused a minor delay in the meiotic progression of wild type cells. However, within 10 hours, the percentage of wild type cells completing MII exceeded 80% (data not shown). Taken together, data from *clb5 clb6 IME2-CLB5* and *clb5 clb6 MET3-CLB5* cells demonstrate that *de novo* synthesis of *CLB5* during sporulation is required for efficient premeiotic S-phase and meiotic progression. No carry over of *CLB5* was observed from the previous mitotic cell cycle, and no problems in mitotic chromosome metabolism were apparent in *clb5 clb6* cells. The inability of these double mutants to initiate premeiotic DNA replication is most likely due to the lack of S-phase-promoting cyclin activity.

IV.2.b. – The transcriptional profile of *CLB5* during sporulation: *CLB5* displays a distinct transcriptional profile during meiotic development. Upon meiotic induction, *CLB5* mRNA is detectable within 2 hours into sporulation (Figure IV-1A, Wild Type). This early expression is coincident with the onset of premeiotic S-phase, and is dependent on the induction of meiosis (see Chapter III). Further into the meiotic program, a peak accumulation of *CLB5* transcript is seen between 5 to 8 hours, corresponding to the onset of middle sporulation (Figure IV-1A, Wild Type). In cells lacking *MBP1*, which encodes the DNA binding subunit of the MBF, meiotic *CLB5* expression was not altered (Figure IV-1A, Δ*mbp1*). *mbp1* mutants displayed no defects in premeiotic S-phase or meiotic recombination (see Chapter III), further demonstrating that *CLB5* expression was not affected in these cells. The meiosis-specific transcription factor Ndt80, a key regulator of middle gene expression, is known to regulate the expression of *CLB*s during sporulation (8). The expression of *NDT80* has been fairly well characterized, demonstrating a pre-middle pattern of expression (28). As well, Ndt80 protein accumulation is most significant during the middle sporulation, consistent with its defined activity and coincident with the peak expression of *CLB5* (Figure IV-1B, Ndt80-HA). Inactivation of *NDT80* abolished this peak *CLB5* expression, leaving behind a constitutive accumulation of *CLB5* transcript (Figure IV-1A, Δ*ndt80*). Though *CLB5* expression is dramatically altered in *ndt80* cells, this cyclin is still induced during early stages and efficient premeiotic DNA replication is observed (see Chapter III). Inactivation of *MBP1* in *ndt80* mutants does not produce any further defects to *CLB5* expression, ruling out even subtle influences of Mbp1 on *CLB5* accumulation (Figure IV-1A, Δ*ndt80* Δ*mbp1*). This data also strongly suggests the existence of other factors inducing the expression of *CLB5* during sporulation.

IV.2.c. – A mutational analysis of the *CLB5* promoter: Sequence scanning of the *CLB5* promoter revealed a number of potential regulatory elements. A cluster of putative MCB elements (MBF binding sites) have been identified based on consensus matching (12, 32) (Figure IV-2A, see *CLB5* promoter schematic). Also, a potential MSE (Middle Sporulation Element) was revealed, possibly serving as an Ndt80 binding site (8). These recognized regulatory elements fall within a 180bp region of the *CLB5* upstream sequence, approximately 400bp from the initiation codon.

To analyze the significance of these identified response elements and other potential regulatory sequences in this region, different mutations were created within the *CLB5* promoter, and these "promoter constructs" were fused to a *clb5* reporter gene. This *clb5* reporter gene contains an internal sequence deletion within the *CLB5* open reading frame, producing a non-functional protein product. Figure IV-2 diagrams all the various constructs created from this promoter (for experiments detailing Ndt80-mediated expression of *CLB5*, and the importance of the proposed MSE, see Chapter V). The expression of these reporter genes was monitored in wild type cells by Northern blot analysis. Due to a distinct mobility of the *clb5* reporter transcript relative to the endogenous *CLB5* transcript, reporter expression was easily detectable (Figure IV-2; *clb5* reporter mRNA migrates slower than the endogenous *CLB5* mRNA, as indicated in the Northern blots presented). *clb5* reporter gene expression under the regulation of the wild type promoter construct displayed a pattern of expression consistent with that of the endogenous *CLB5* mRNA accumulation in sporulating cells (Figure IV-2A). To determine the transcriptional influence of these recognized regulatory elements, we analyzed the expression of the *clb5* reporter transcript from two promoters containing sequence deletions of this region. In the Δ179 construct, a deletion of sequence between -222 to -398 relative to the ATG start codon removed most of the identified regulatory elements within the *CLB5* promoter (Figure IV-2B, Δ179 schematic). A significantly reduced level of *clb5* reporter expression was seen from this Δ179 promoter in sporulating cells (Figure IV-2B, *clb5*). Though a putative MCB remained in this construct (MCB1), this had a very minor effect, if any, on the reporter expression observed. Also, no apparent middle sporulation expression was seen from this deletion promoter, consistent with the elimination of the putative MSE at position -230 (for more details see Chapter V). Results from the Δ179 promoter suggest that all the major meiosis-specific regulation of *CLB5* is derived from the sequence deleted in this construct. This has allowed us to designate this region between -222 and -398 as the *CLB5* Upstream Activating Sequence (UAS^{CLB5}). *clb5* reporter expression from the Δ178 promoter was also significantly reduced compared to wild type (Figure IV-2C, *clb5*). However, expression from this promoter displayed a slightly regulated pattern of transcription, with an accumulation coincident with that of the middle sporulation genes *SPS1* and *SPS2* (Figure IV-2C, *SPS1,2*). This pattern may be consistent with the presence of a putative MSE in Δ178 (Figure IV-2C, Δ178 schematic), however accumulation of reporter transcript from this promoter was significantly reduced compared to that of the wild type promoter (Figure IV-2, compare *clb5* from C and A). It is not apparent why the influence of Ndt80 on the Δ178 promoter was weaker in this construct. This effect may implicate this potential MSE as a weak activator of Ndt80-mediated expression, in which case another, more favorable Ndt80-binding site may reside within the UAS^{CLB5} (see Chapter V).

Even though we have demonstrated that Mbp1 does not appear to influence the meiotic expression of *CLB5*, we proceeded to scrutinize the potential MCBs in UAS^{CLB5} to investigate whether or not these sites are active regulatory elements during meiotic development. Site-specific mutagenesis was used to inactivate a number of these MCBs. The Δmcb4 promoter contains mutations in 4 of 6 MCBs at positions indicated in the schematic of this construct (Figure IV-2D, Δmcb4 schematic). The Δmcb5 promoter contains mutations in 5 of 6 MCBs identified (Figure IV-2E, Δmcb5 schematic). In both cases, inactivation of the putative MCB elements produces very minor effects on the expression profile of the *clb5* reporter gene relative to the endogenous *CLB5* expression (Figure IV-2D and -2E; compare *clb5* to *CLB5*). However, reporter expression driven by Δmcb5 appears to be reduced compared to reporter expression derived from Δmcb4. Interestingly, this data suggests that MCBs within the *CLB5* promoter may be imposing some transcriptional influence on the meiotic expression of this gene. However, this reduction is only minor compared to the significantly decreased expression seen in the Δ178 and Δ179 promoters. Therefore, these data also demonstrate the existence of other unrecognized regulatory elements within the UAS^{CLB5} regulating *CLB5* expression.

To determine if the *CLB5* promoter mutations described above might have any physiological consequences, we generated constructs in which a functional *CLB5* open reading frame was placed under the regulation of the various promoters outlined in Figure IV-2. These new promoter constructs were introduced into haploid *clb5 clb6* mutants, providing the only source of *CLB5* for these cells. Homozygous diploids were created for each strain, and these cells were analyzed for *CLB5* expression and sporulation phenotypes. In most cases, Northern blot analysis of *CLB5* expressed from these promoters in *clb5 clb6* cells was consistent with the *clb5* reporter expression seen in wild type cells. *CLB5* expression driven by the wild type promoter construct demonstrated a consistent pattern of expression, with an early induction followed by a peak accumulation a 5 to 8 hours into sporulation (Figure IV-3A *CLB5*). Both Δ178 and Δ179 promoters displayed a profound reduction in *CLB5* expression relative to the wild type construct (Figure IV-3A, Δ179 and Δ178). However, much to our surprise, inactivation of the MCBs in the *CLB5* promoter resulted in a significant defect in *CLB5* transcript expression (Figure IV-3A, Δmcb4 and Δmcb5). Most notably, we observed a significantly altered pattern of expression from the Δmcb4 and Δmcb5 promoters. Both initiate a gradual increase in *CLB5* transcript abundance, definitely distinct from the regulated wild type pattern of expression (Figure IV-3A, compare Δmcb4 and Δmcb5 to *CLB5* wild type). Also, the level of expression driven by the Δmcb5 promoter was noticeably reduced compared to that driven by the wild type promoter (Figure IV-3A, Δmcb5). Quantitation of the meiotic-*CLB5* expression from these various promoters clearly demonstrated this altered expression profile and reduced mRNA abundance (Figure IV-3B). Relative meiotic *CLB5* mRNA levels from the wild type, Δmcb4, Δmcb5 and Δ178 promoters in *clb5 clb6* cells, at 1, 4, 6 and 12 hours post-induction are presented. Relative *CLB5* abundance from the wild type promoter displayed the regulated pattern of transcript accumulation expected (Figure IV-3B, *CLB5*). An obviously altered profile was seen for Δ178, with noticeably reduced levels of *CLB5* at most time-points (Figure IV-3B, Δ178). A similar altered pattern was seen for the Δmcb5 promoter, in which 5 of 6 MCBs are inactivated (Figure IV-3B, Δmcb5). Relative *CLB5* accumulation from the Δmcb4 promoter, where 4 of 6 MCBs are inactivated, also displays reduced expression at certain time-points (Figure IV-3B, Δmcb4). In fact, a greatly delayed peak accumulation of transcript is seen in both Δmcb4 and Δmcb5, which is consistent with their observed expression seen in the Northern blots presented in Figure IV-3A. This data distinctly deviates from the minor effects we observed in the *clb5* reporter expression driven by promoters containing the MCB mutations (Figure IV-2D and -2E), and may suggest that a more complicated regulatory mechanism regulates *CLB5* induction during sporulation (see discussion below).

A common feature of most MCB-mediated expression is a clustering of this regulatory element in the promoters of genes it regulates (18). How this clustering contributes to the efficiency of transcription is not well documented. Through our analysis of the MCBs in the *CLB5* promoter, we have seen that inactivating 5 of 6 MCBs produces a greater reduction in transcription than when we mutate 4 out of 6 MCBs. This implicates MCB elements as potential sites of transcriptional activation within the *CLB5* promoter. This also seems to suggest that MCB-clustering might serve to impart a cooperative or additive mechanism to the transcriptional activation of this promoter. Due to the systematic method we used to inactivate the MCBs within the *CLB5* promoter, it may be possible that the fifth MCB at position -389 (MCB2) (Figure IV-2A) is particularly active in promoting transcription, and inactivation of this site may have produced the major defects seen in the Δmcb5-*CLB5* expression. To investigate this possibility, we created a new *CLB5* promoter construct carrying a single mutation in MCB2 (Figure IV-3A, Δmcb1). Inactivation of this single MCB displayed no defects in *CLB5* expression; this expression followed a profile closely resembling that of the wild type promoter (Figure IV-3A, compare Δmcb1 to wild type *CLB5*). Therefore this single MCB was not responsible for the reduced level of *CLB5* expression seen in Δmcb5, suggesting that the MCBs may be working in a cooperative or additive manner to induce *CLB5* transcription. This proposed mechanism may even be extended towards other MCB-regulated genes that carry such clustering in their promoters.

The strikingly contradictory results between the *clb5* reporter gene expression in wild type cells, and the functional *CLB5* expression in *clb5 clb6* cells from the Δmcb4 and Δmcb5 promoters, raises some intriguing possibilities regarding the transcriptional regulation of this S-phase cyclin. As described above, expression from the Δmcb5 promoter in *clb5 elb6* cells produces significant defects in the accumulation of *CLB5*, while only minor defects are seen in the expression of the Δmcb5-*clb5* reporter gene in wild type cells. One possible explanation for this observation could be that Clb5 might be promoting the activation of its own promoter through a positive-feed back loop. If this is the case, this positive feed-back mechanism must be independent of the MCB-induced transcription. Therefore, this result implicates the existence of other regulatory sequences within the *CLB5* promoter, which may be targeted by unknown regulators of this gene. The notion of a feed-back loop involved in cyclin expression is not completely unheard of. The mitotic cyclin Clb2 has been shown to affect its own transcription by phosphorylating the transcriptional activator Ndd1, which stabilizes the Ndd1/Fkh2/Mcm1 transcription factor complex, thereby enhancing its own expression (29). However, this mechanism acts on the primary machinery influencing the transcription of *CLB2*, whereas Clb5 may be influencing secondary or parallel transcriptional activators of its own expression.

Though we strongly believe that our results concerning these mutant promoters are due to the inactivation of putative MCB elements within the UAS^{CLB5}, it is important to note that mutations we have introduced may have inadvertently affected other unrecognized regulatory elements within this sequence. To this end, we have scanned the *CLB5* promoter sequence further to search for other known transcription factor binding sites, however none were obviously apparent.

IV.2.d. – Inactivation of MCBs in the *CLB5* promoter affects the accumulation of Clb5-associated kinase activity and produces defects in premeiotic S-phase and sporulation efficiency: Since the activity of Clb5-

Cdc28 is essential to the progression of sporulation, specifically at premeiotic S-phase, we next analyzed the histone H1 kinase activity associated with Clb5 in sporulating cells. *CLB5* expressed from the wild type promoter construct in *clb5 clb6* cells produced an associated kinase activity profile that closely resembles the pattern of mRNA accumulation observed (Figure IV-4A, *CLB5*). These same cells initiated premeiotic DNA replication in a timely fashion, beginning at approximately 2 hours into sporulation, and completing S-phase at about 4 hours (Figure IV-4B, *CLB5*). It is important to note that the detectable level of Clb5-associated kinase activity seen within 2 hours of the meiotic-induction is the level of relevant activity required for the activation of premeiotic DNA replication. Histone H1 kinase activity associated with Clb5 from *clb5 clb6* cells containing the Δmcb4-*CLB5* construct displayed a significantly altered profile relative to the wild type activity (Figure IV-4A, Δmcb4). A gradual increase in Clb5-associated kinase activity was detected in these cells, resulting in a significant shift in the peak accumulation of activity. This kinase profile is consistent with the *CLB5* mRNA expression observed from this Δmcb4 promoter construct (Figure IV-3, Δmcb4). Interestingly, the initiation of premeiotic DNA replication in these *clb5 clb6* Δmcb4-*CLB5* cells is significantly delayed, beginning at 4 hours into sporulation (Figure IV-4B, Δmcb4). This delay in the initiation of premeiotic S-phase is most likely due to the reduced level of Clb5-associated kinase activity seen at early times in these cells (Figure IV-4A, Δmcb4). Eventually, these cells do accumulate the relevant level of activity necessary to initiate S-phase (after 4 hours), producing the observed delay. Though these cells appear to complete

DNA replication, the duration of S-phase seems significantly elongated as well, possibly indicative of limiting Clb5-associated kinase activity (Figure IV-4B, Δmcb4). Consistent with the dramatically reduced expression of *CLB5* from the Δmcb5 promoter in *clb5 clb6* cells, very low levels of detectable Clb5-associated kinase activity is also observed throughout sporulation in these cells (Figure IV-4A, Δmcb5). As a result these cells do not reach the relevant levels of activity required to initiate DNA replication (Figure IV-4B, Δmcb5).

Based on the observed defects in *CLB5* expression and DNA replication seen from cells expressing the various constructs described above, we next wanted to evaluate what effects these promoter mutations have on sporulation efficiency. Homozygous *clb5 clb6* diploids expressing *CLB5* from the wild type promoter construct were able to complete both meiotic divisions and achieve a wild type level of tetrad formation (Figure IV-4C, *CLB5*). As expected, cells carrying the Δ178 promoter construct do not rescue the *clb5 clb6* defect and were unable to sporulate (Figure IV-4C, Δ178). Cells carrying the Δmcb1 construct, containing a mutation in one MCB element, were able to sporulate to levels comparable to cells harboring the wild type promoter construct (Figure IV-4C, Δmcb1). In contrast to Δmcb1, cells carrying the Δmcb4 promoter construct demonstrated a dramatic reduction in sporulation efficiency, with only 30% of cells completing MII (Figure IV-4C, Δmcb4). A more severe sporulation defect was seen in cells carrying the Δmcb5 promoter construct, with less than 5% of cells completing MII (Figure IV-4C, Δmcb5). Therefore relative sporulation efficiencies seen for cells harboring these *CLB5* promoter constructs appear to correlate with the degree of *CLB5* expression detected, and are indicative of the cells ability to successfully complete S-phase.

IV.2.e. – *CLB5* promoter mutations produce altered cellular morphology:

Since proliferating *clb5 clb6* mutants are delayed in the initiation of DNA replication, these cells display a lengthened G_1-phase. This means the cells undergo a longer period of growth before S-phase resulting in a larger cell volume and elongated morphology (Figure IV-5, compare A and B). Introduction of the wild type promoter construct expressing *CLB5* completely rescued this mitotic *clb5 clb6* phenotype (Figure IV-5C). This clearly indicates that functionally relevant expression of *CLB5* is achieved from this wild type promoter. The Δ178 promoter construct did not rescue this *clb5 clb6* mitotic defect, with cells still demonstrating an elongated morphology and a greater cell volume (Figure IV-5D). This is consistent with a dramatically reduced level of *CLB5* expression seen from this promoter, and implicates important mitotic regulatory sequences within the UAS^{CLB5} as well. The Δmcb4 promoter, in which 4 of 6 MCBs were inactivated, still supported adequate expression of *CLB5*, as indicated by the almost complete rescue of the *clb5 clb6* phenotype (Figure IV-5E). These cells may not be as defective in initiating timely DNA replication, however a slight increase in cell volume is observed. In striking contrast, cell bearing the Δmcb5-*CLB5* construct, in which 5 of 6 MCBs are inactivated, are completely unable to rescue the *clb5 clb6* defect (Figure IV-5F). Consistent with the meiotic defect, this indicates that the Δmcb5 promoter does not express the necessary levels of *CLB5* required to promote effective DNA replication during growth. Indeed, kinase activity purified from asynchronous proliferating Δmcb5 cells was dramatically reduced compared to that of cell carrying the wild type construct (Figure IV-4A). Interestingly, effects of these promoter mutations in proliferating cells appear to be consistent with those effects seen in sporulating cells. This might suggest that some parallels may exist between the regulation of this cyclin during these two processes.

IV.2.f. – MCB elements are important regulatory sequences in the *CLB5* promoter: The most striking result from our analysis of the *CLB5* promoter is that, even though Mbp1 is not required for the meiotic regulation of *CLB5*, the cluster of MCB elements within the UAS^{CLB5} is absolutely necessary for the early expression of this S-phase cyclin. It has been demonstrated that MBF-regulated genes (such as *RNR1* and *TMP1*) lose their periodic expression upon deletion of *MBP1* (19). Therefore, the MBF was thought to act upon MCBs as a transcriptional regulator and not necessarily a transcriptional activator. However, there is evidence to support a greater complexity to MCB-mediated regulation. Deletion of MCBs within the promoters of MBF-regulated *TMP1* and *POL1* essentially eliminates their transcriptional expression (14, 23), suggesting that MCBs may be required for the activation of these genes. Our data also support this result, implicating the MCBs within the *CLB5* promoter as sites of transcriptional activation and not just regulators of gene periodicity. To demonstrate the effectiveness of MCB elements as transcriptional-activator sequences in sporulation, we created constructs in which the *CLB5* open reading frame was placed under the regulation of a single wild type MCB site (MCB) or a single mutated MCB site (mcbx). These sites were actually incorporated into the Δ179-*CLB5* promoter construct. Although this Δ179 promoter contains one core MCB site, our analysis has shown that this site is insufficient to promote *CLB5* expression on its own (Figure IV-2B). When introduced into *clb5 clb6* mutants, the constructs regulating *CLB5* from a wild type MCB induced the meiotic expression of *CLB5* mRNA to levels significantly greater than that derived from the construct containing the mutant MCB (mcbx) (Figure IV-6A, top graph). Interestingly, the increased level of *CLB5* expression from the wild type MCB supported an increased rate of sporulation compared to the strongly defective phenotype of the mcbx-*CLB5* cells (Figure IV-6B, compare MCB to mcbx for the *MBP1/MBP1* data set) - about 50% of the MCB-*CLB5* cells completed sporulation, while only 3% of the mcbx-*CLB5* cells were able to form tetrads. We next wanted to investigate if Mbp1 was influencing the expression of *CLB5* from these single-MCB promoters. We introduced both these constructs into *clb5 clb6 mbp1* mutants and monitored *CLB5* expression during sporulation in these cells. *CLB5* expressed from the wild type MCB accumulated to higher levels throughout sporulation than did *CLB5* expressed from the mutant mcbx (Figure IV-6A, bottom graph). This clearly suggests a distinct transcriptional activation of this MCB independent of Mbp1. We also noticed that at early time-points, the relative level of *CLB5* mRNA from the wild type MCB and mutant mcbx promoters was comparable to their respective expression in *MPB1* cells (Figure IV-6A, compare 0-5 hrs for MCB-*CLB5* and mcbx-*CLB5* in both strains). However, expression from both these constructs was noticeably elevated in *MPB1* cells at later time-points compared to that seen in *mpb1* mutants (Figure IV-6A, compare 5-12 hrs for MCB-*CLB5* and mcbx-*CLB5* in both strains). Whether this effect has to do with an indirect influence of other Mbp1-regulated genes, or whether Mbp1 may be influencing this dramatically altered *CLB5* promoter is not clear. Nevertheless, these *clb5 clb6 mbp1* cells carrying MCB-*CLB5* demonstrated a sporulation efficiency of about 50%, significantly greater than the sporulation efficiency seen for the same cells carrying mcbx-*CLB5* (12%), and consistent with the results in the *MBP1* strain (Figure IV-6B). It is unclear why greater sporulation efficiency is seen in *clb5 clb6 mbp1* cells carrying the mcbx-*CLB5* (12%) than that seen in *clb5 clb6 MBP1* cells (3%).

IV.2.g. – *mbp1* mutants express specific MCB-binding activity during meiotic development: Our data clearly indicates that MCB elements play an important role in activating the transcriptional expression of *CLB5* during meiotic development. And, interestingly, these MCBs activate *CLB5* expression independently of Mbp1 activity. Our work in this matter has raised intriguing implications regarding the MCB and the regulation of *CLB5* during meiotic development. Most strikingly, unique mechanisms appear to be targeting *CLB5* during early sporulation, promoting its essential S-phase expression. Indeed, we have shown that *de novo* expression of *CLB5* during sporulation does occur, and is required. This may have supported the development of alternate mechanisms to express *CLB5* in both these cell fates. Unique meiotic mechanisms regulating *CLB5* may involve the activity of novel factors targeting MCBs in the UAS^{CLB5} during sporulation. An MCB-binding activity distinct from Mbp1 has been previously observed in mitotically growing cells. A 17kDa protein in cellular extracts was shown to demonstrate affinity for the *MluI* motif (MCB core) (36). However, this data has not been followed up. We have also conducted preliminary experiments in search of novel MCB binding factors. Through electrophoretic mobility shift assay (EMSA), we have been able to identify a specific MCB-binding activity in sporulating cells (Figure IV-7). A specifically-shifted species was clearly detected in both wild type and *mbp1* extracts derived from cells undergoing meiotic development (Figure IV-7A, lanes 3 and 8). This specifically-shifted species was effectively competed by excess unlabeled wild type MCB probe (Figure IV-7A, lanes 4 and 9; Figure IV-7B, see corresponding quantitated data). A less effective competition was observed by excess unlabeled mutant MCB probe (mcbx) (Figure IV-7A, lanes 5 and 10; Figure IV-7B, see corresponding quantitated data). This data clearly demonstrates a specific MCB-binding activity in *mbp1* cells, and strongly implicates the existence of a novel MCB-binding factor in sporulating cells. An interesting potential exists for this novel factor regulating other MCB-driven genes during sporulation. If this can be determined, it is possible that subclasses of MCB-containing genes may become evident. To date, only two proteins have been shown to bind to MCB elements: Mbp1 and Swi4. This binding activity is unlikely to be Swi4, since we have confirmed that this transcription factor is immediately degraded upon meiotic induction (see Chapter III). Several candidate MCB-binding proteins sharing sequence similarity to the DNA-binding domain of Mbp1 are encoded in *Saccharomyces cerevisiae* (*SOK2*, *PHD1*, and *GAT1*). However, there is no evidence to implicate any of factors as potential regulators of MCB-containing genes. Interestingly, work done in *Schizosaccharomyces pombe* analyzing the regulation of the S-phase gene *cdc18*$^+$ has revealed that, along with the DSC1 complex (MBF in *S. pombe*), a novel MCB-specific transcriptional complex also binds to the promoter of this MCB-containing gene (17). Additionally, DSC1 only appears to play a regulatory role on the expression of *cdc18*$^+$, and binding of DSC1 to this gene promoter does not correlate with its transcriptional activation. Whether this novel MCB-specific transcriptional complex is involved in the activation of *cdc18*$^+$ has not been determined.

It may be reasonable to assume that variations in MCB motifs, including surrounding sequences, may influence the regulation of MCB elements. These flanking sequences may provide the targeting required for a meiosis-specific reprogramming of these regulatory sites. The actions of potential meiosis-specific factors and unique alterations of a subset of MCB-containing promoters may set the stage for the differential regulation of genes during sporulation (eg. *CLB5*). The importance of sequences surrounding a core regulatory element has been reported previously for the meiosis-specific transcription factor Ndt80. Distinct influences by sequences flanking the core MSE have been shown to be important in determining Ndt80 binding affinity and associated transcriptional activity (30). Also, these flanking sequences prove to be important in balancing the competitive interaction between Ndt80 and its specific transcriptional repressor Sum1. In *S. pombe*, a study on the regulation of the MCB-containing gene *cdc18*$^{+}$ has revealed that the orientation and organization of MCBs play an important role in the expression of this gene (17). Significant influences by surrounding sequences (inverted repeats and a spacer region) also appear to make essential contributions to *cdc18*$^{+}$ expression. These flanking sequences may be important for enhancing the transcriptional activation of genes by promoting interactions between potential co-regulators, thereby producing a higher order transcriptional complex (6).

Recently, through a genome wide screen, *CLB5* has also been observed to be targeted by the RSC nucleosome remodeling complex (26). RSC is the most abundant nucleosome remodeling activity in yeast and is essential for growth (4). The RSC complex has also been shown to be required for sporulation. Temperature sensitive mutants of *STH1*, a subunit of RSC, display a decrease in the expression of early sporulation genes, and also show poor spore formation (37). Also, other components of RSC, *RSC1* and *RSC2*, may have roles in meiotic development. *RSC1* and *RSC2* have actually been shown to be required for the expression of mid-late sporulation-specific genes (3), and the deletion of either *RSC1* or *RSC2* causes a decrease in sporulation efficiency and abnormal spore morphology (38). Interestingly, *RSC1* and *RSC2* encode mutually exclusive components of the RSC complex (5). It has been suggested that this might be indicative of distinct forms of the RSC. Since RSC functions during mitotic proliferation and meiotic development, it may be possible that meiosis-specific variations of this complex may promote the differential regulation we have uncovered for *CLB5*. However, there is no evidence to support this supposition.

Although we have demonstrated an important role for the MCBs in the expression of *CLB5* during meiotic development, our data also suggest the existence of other as-of-yet unidentified regulatory sequences within the *CLB5* promoter. None of the alterations we have made to this promoter has produced a complete elimination of *CLB5* expression. Also, inactivating 5 of 6 MCB elements in the UAS^{CLB5} appears to have almost no effect on the expression of the *clb5* reporter gene in wild type cells. Together, this indicates that both within the UAS^{CLB5}, and in sequences surrounding this region, other regulatory elements must exist to facilitate this expression. These potential sites might be involved in an initial induction of *CLB5* upon entry into sporulation, as well as the proposed positive feed-back loop that may be influencing early *CLB5* accumulation. It is well known that many meiosis-specific genes lack any recognizable regulatory sequences involved in meiotic-transcription (7), and so other unknown sites and factors may exist to support this regulation.

IV.2.h. – Conclusion: Our analysis of *CLB5* expression during meiotic development reveals an intricate and specifically coordinated regulation for this S-phase cyclin. In contrast to mitotic growth, the meiotic regulation of *CLB5* is independent of Mbp1. However, MCB elements within the *CLB5* promoter are crucial for the essential expression of this cyclin during premeiotic S-phase. *CLB5* may also be involved in a positive feedback mechanism enhancing the expression of its own promoter. We also demonstrate the existence of a novel MCB-binding factor expressed in sporulating cells, which may be activating the expression of *CLB5* during sporulation. This work strongly counters the more simplistic models previously suggested for *CLB5* transcriptional regulation. *CLB5* serves as an excellent example of how a cell-cycle regulated gene becomes reprogrammed to function during meiotic development. A differential requirement for Mbp1, possible novel regulatory sequences, and the undoubted involvement of unknown meiosis-specific factors all combine to support the essential role of *CLB5* in sporulation.

IV.3 - References

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Table IV-1
Effects of *CLB5* expression on meiosis and spore formation

Genotype	Growth medium	Sporulation medium	% cells completed MII at 12 hrs	% asci 24 hrs	% spore viability
CLB5 CLB6	YEP-KAc	SPM	96	87	99
clb5 clb6	YEP-KAc	SPM	18	0	0
clb5 clb6 IME2-CLB5	YEP-KAc	SPM	95	83	96
clb5 clb6 MET3-CLB5	SD-MET	SPM+MET	24	10	72
clb5 clb6 MET3-CLB5	SD+MET	SPM	72	67	97

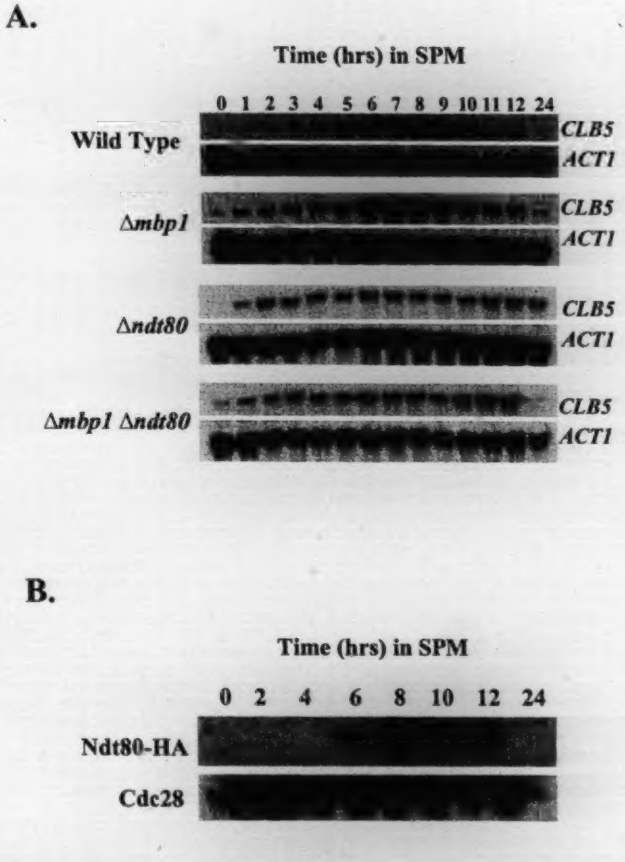

A.
Time (hrs) in SPM
0 1 2 3 4 5 6 7 8 9 10 11 12 24
Wild Type
CLB5
ACT1
Δmbp1
CLB5
ACT1
Δndt80
CLB5
ACT1
Δmbp1 Δndt80
CLB5
ACT1
B.
Time (hrs) in SPM
0 2 4 6 8 10 12 24
Ndt80-HA
Cdc28

Figure IV-1: The meiotic expression of *CLB5* is dependent on *NDT80* but not *MBP1*. (A) Homozygous diploid cells, either Wild Type or containing the deletion(s) indicated to the left were induced to sporulate. Samples collected at indicated time-points were processed for the extraction of RNA and subjected to Northern blot analysis. Probing for *CLB5* mRNA reveals the transcriptional profile for this cyclin in each strain undergoing meiotic development. *ACT1* transcripts were probed as a loading control. Note, this Northern data has been presented in Chapter III (Figure III-1). (B) Homozygous diploid cells expressing Ndt80-HA were induced to sporulate, and protein samples collected at time-points indicated were analyzed by Western blot. Blots were probed for the HA epitope and for Cdc28 (anti-PSTAIRE) as a loading control.

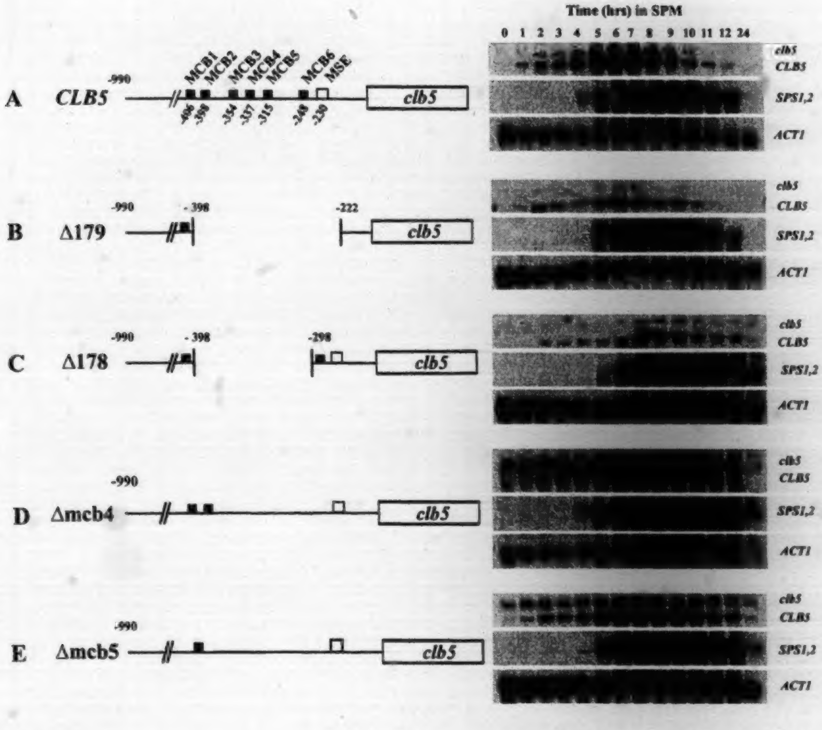

Time (hrs) in SPM
0 1 2 3 4 5 6 7 8 9 10 11 12 24
A
CLB5
-990
MCB1
MCB2
MCB3
MCB4
MCB5
MCB6
MSE
-408
-398
-354
-337
-315
-248
-230
clb5
B
Δ179
-990
-398
-222
C
Δ178
-298
D
Δmcb4
E
Δmcb5
clb5
CLB5
SPS1,2
ACT1

Figure IV-2: Sequence encompassing a 180bp region of the *CLB5* promoter supports the majority regulation of *CLB5* transcriptional expression during meiotic development. Wild type homozygous diploids carrying various *clb5* reporter constructs were induced to sporulate, and RNA samples collected at various time-points were analyzed by Northern Blot for *CLB5, SPS1,2* and *ACT1* mRNA. The reporter transcript (*clb5*) migrates more slowly than the endogenous *CLB5* mRNA (*CLB5*). Promoter schematics for wild type and mutant *clb5* reporter constructs are presented at left corresponding to their respective Northern blot data; putative MCB elements (■) and a potential MSE site (□) are indicated, and their relative positions with respect to the *CLB5* start codon are shown in the wild type (*CLB5*) promoter schematic. (A) *CLB5*, wild type promoter. (B) Δ179 promoter, which contains a deletion from -222 to -398 relative to the start codon. (C) Δ178 promoter, which contains a deletion from -298 to -398. (D) Δmcb4 promoter, which has mutations in MCB3, MCB4, MCB5 and MCB6. (E) Δmcb5, which has mutations in MCB2, MCB3, MCB4, MCB5 and MCB6.

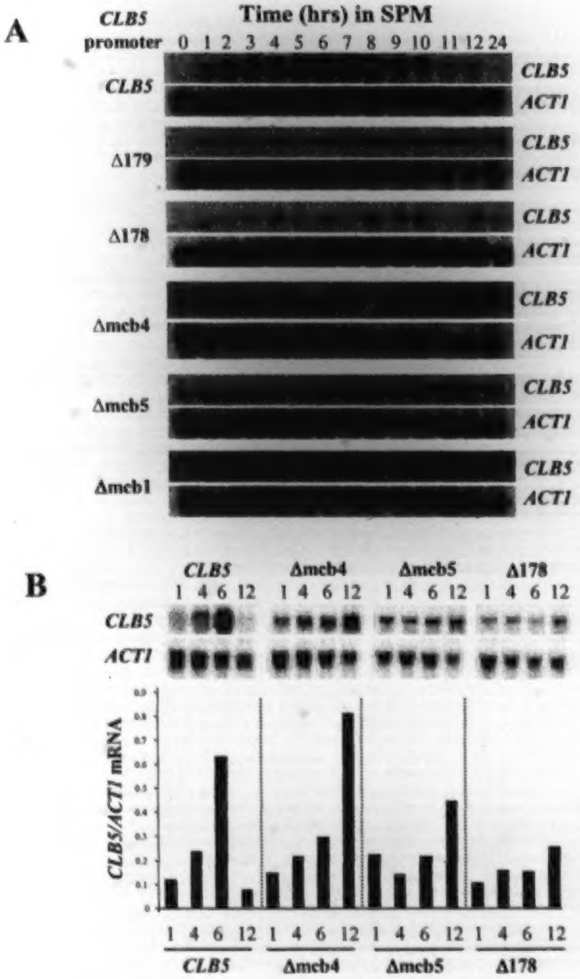


Figure IV-3: Mutating MCBs in the *CLB5* promoter reduces *CLB5* mRNA levels throughout sporulation. (A) Homozygous *clb5 clb6* diploids expressing *CLB5* from wild type or mutant *CLB5* promoter constructs (as presented in Figure IV-2), were induced to sporulate and RNA samples collected were subjected to Northern blot analysis, probing for *CLB5* and *ACT1*. (B) RNA samples from sporulating *clb5 clb6* diploids (from time-points indicated) expressing *CLB5* from wild type (*CLB5*), Δmcb4, Δmcb5 or Δ178 promoters were analyzed on a single Northern blot. The blot was probed for *CLB5* and *ACT1*. ^{32}P-labeled signals were quantitated by a phosphorimager, and relative *CLB5/ACT1* mRNA are plotted in corresponding histograms below.

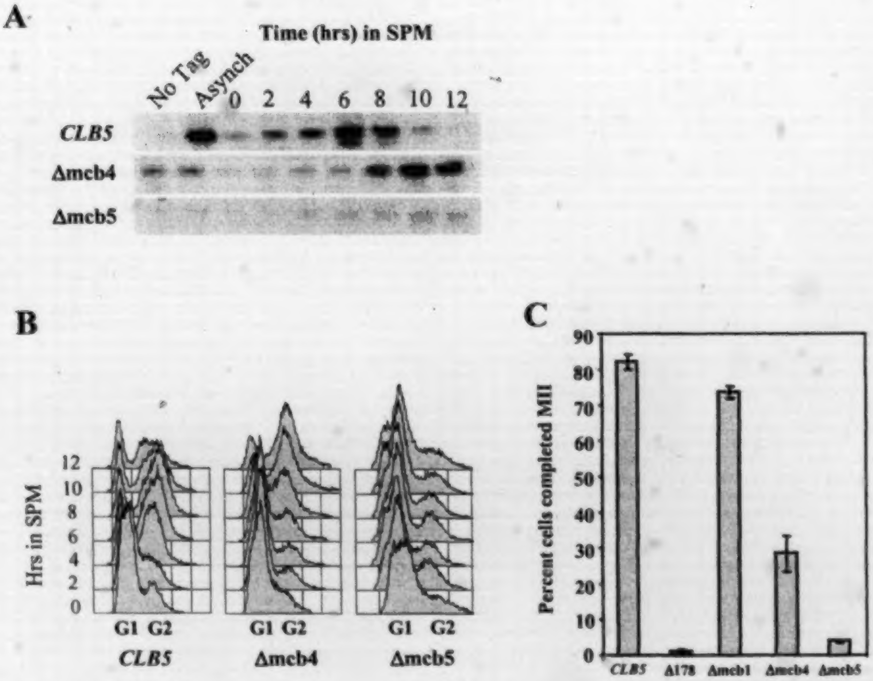
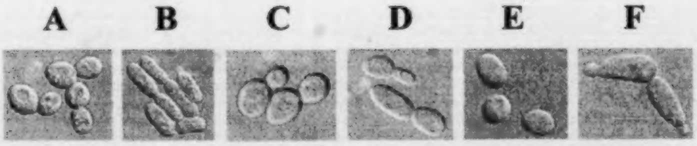


Figure IV-4: Mutating MCBs in the *CLB5* promoter reduces Clb5-associated kinase activity throughout sporulation, produces defects in premeiotic S-phase, and reduces sporulation efficiency. (A) Homozygous *clb5 clb6* diploids expressing an HA tagged allele of *CLB5* from wild type, Δmcb4 or Δmcb5 promoter constructs were induced to sporulate, and samples were collected at time-points indicated to analyze the Clb5-associated histone H1 kinase activity. Protein extracts from each sample were subjected to immunoprecipitation with anti-HA antibodies to purify Clb5-associated kinase activity. Extracts from asynchronously growing *clb5 clb6* diploids carrying the indicated constructs (Asynch), or carrying an untagged construct (No Tag) were also analyzed. (B) Homozygous *clb5 clb6* diploids expressing *CLB5* from the wild type, Δmcb4 or Δmcb5 promoter construct were induced to sporulate and samples were collected at the indicated time-points for analysis of DNA content by flow cytometry. G1 (2C) and G2 (4C) peaks are indicated for each overlay plot. (C) Homozygous *clb5 clb6* diploids carrying the indicated promoter constructs expressing a *CLB5* open reading frame were induced to sporulate. After 24 hours in SPM, the percentage of cells that had completed two meiotic divisions (indicated by more than two DAPI-staining chromatin masses) was scored.



	A	B	C	D	E	F
Strain	***CLB5/CLB5*** ***CLB6/CLB6***	***clb5/clb5*** ***clb6/clb6***	***clb5/clb5*** ***clb6/clb6***	***clb5/clb5*** ***clb6/clb6***	***clb5/clb5*** ***clb6/clb6***	***clb5/clb5*** ***clb6/clb6***
Construct	**vector**	**vector**	***CLB5***	**Δ178-*CLB5***	**Δmcb4-*CLB5***	**Δmcb5-*CLB5***
Mean cell Volume (fl)	74.39	114.64	74.67	105.40	82.29	100.98

Figure IV-5: Mutating MCBs in the *CLB5* promoter alters cell morphology in growing strains. (A-F) Homozygous diploids of the indicated genotype and carrying the indicated constructs were grown in YEPD to mid-log phase. Images were captured of representative cells in the asynchronous populations. (A) Wild type *CLB5 CLB6* diploids transformed with empty vector. (B) *clb5 clb6* diploids transformed with empty vector. (C) *clb5 clb6* diploids transformed with the wild type promoter construct expressing *CLB5*. (D) *clb5 clb6* diploids transformed with the Δ178 promoter construct expressing *CLB5*. (E) *clb5 clb6* diploids transformed with the Δmcb4 promoter construct expressing *CLB5*. (F) *clb5 clb6* diploids transformed with the Δmcb5 promoter construct expressing *CLB5*.

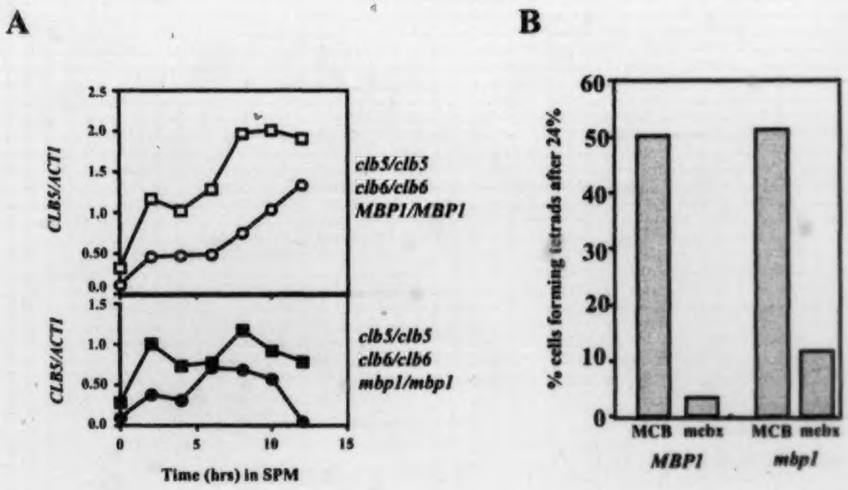


Figure IV-6: MCB sequences are sufficient to drive transcriptional expression during sporulation. (A) Homozygous *clb5 clb6 MBP1* diploids (open symbols) or *clb6 clb5 mbp1* diploids (solid symbols), carrying a *CLB5* open reading frame regulated by a wild-type MCB sequence (□,■) or a mutant MCB sequence (○,●), were induced to sporulate. RNA samples collected at the indicated time-points were analyzed by Northern blot, probing for *CLB5* and *ACT1*. Signals were quantitated by a phosphorimager. *CLB5* signal is represented relative to *ACT1* throughout the time-course. (B) Homozygous *clb5 clb6 MBP1* and *clb5 clb6 mbp1* diploids expressing *CLB5* from a wild-type MCB (MCB) or a mutant MCB (mcbx), were induced to enter sporulation. After 24 hours the percentage of cells forming asci was determined.

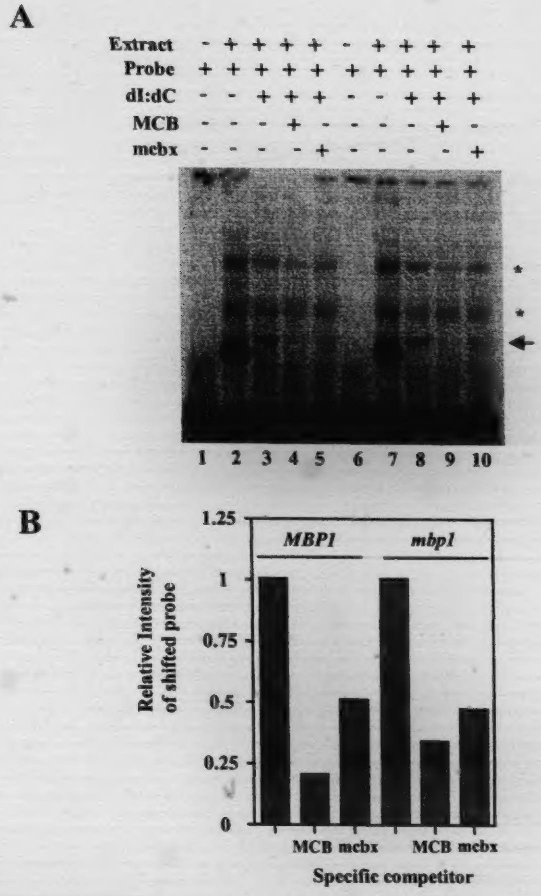


Figure IV-7: Sporulating cells lacking Mbp1 demonstrate specific MCB binding activity. (A) Extracts were prepared from sporulating *MBP1* diploids (lanes 1-5) and sporulating *mpb1* diploids (lanes 6-10), and assayed for MCB-binding activity in a gel mobility shift assay. All lanes contain an equal amount of labeled MCB probe (1pmol). No extract is present in binding assays for lanes 1 and 6. Binding assays in lanes 2 and 7 contain no nucleotide binding competitors. Non-specific nucleotide competitor poly dI:dC is added to binding reactions in lanes 3-5 and 8-10 (2ug/reaction). This amount of poly dI:dC corresponds to a 200X molar excess relative to labeled probe. Reactions in lanes 4 and 9 also contain excess (90pmol) unlabelled wild type MCB competitor (MCB), whereas lanes 5 and 10 contain excess (90pmol) unlabelled mutant MCB competitor (mcbx). The specifically shifted species is indicated with an arrow (←). Two non-specific shifted species were also seen, indicated with asterisks (*). (B) The specifically shifted species in (A) was quantitated by phosphorimager. Signal from lanes 4 and 5 is presented relative to lane 3 (*MBP1* data set), and signal from lanes 9 and 10 is presented relative to lane 8 (*mbp1* data set).

Chapter V

Saccharomyces cerevisiae S-phase Cyclin *CLB5* Contains Two MSEs in Its Promoter that Demonstrate Unequal Transcriptional Activity

A version of this chapter has been submitted for publication:
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V.1 – Introduction

All sexually reproducing organisms undergo a conserved process known as gametogenesis. A unique component of this process is a specialized cell division program called meiosis. Parental diploid cells initiate one round of DNA replication, followed by two consecutive nuclear divisions resulting in haploid progeny. Gametogenesis in the budding yeast *Saccharomyces cerevisiae*, known as sporulation, produces haploid spores, each with the potential to germinate and fuse to haploids of the opposite mating type to regenerate diploid cells. The driving force behind sporulation in yeast is a highly regulated cascade of events supported by the sequential transcription of at least four temporal classes of genes – early, middle, mid-late and late genes (20). A major regulator of early gene expression and a key factor initiating sporulation is the Ime1/Ume6 transcription factor complex. This complex targets a conserved DNA sequence (URS1) found in the promoter regions of many early genes (20, 34). Most early gene products are involved in promoting premeiotic DNA replication, pairing of homologous chromosomes and meiotic recombination (14). Some members of the early gene family encode factors which are involved in the transition into middle sporulation, leading to the induction of the middle genes (7). A key regulator of the middle gene family is the meiosis-specific transcription factor Ndt80, whose activity is necessary for the progression from prophase into meiosis I (7, 40). The products of middle sporulation genes regulate the processes of meiotic nuclear divisions and spore formation, and lead to the expression of mid-late and late genes (6).

The mid-late and late gene families encode factors involved in the final stages of spore morphogenesis (20).

Ndt80 is a member of the Ig-fold famly of transcription factors (9, 16). This transcription factor leads to the expression of greater than 150 middle sporulation genes by binding to a regulatory DNA sequence known as the Middle Sporulation Element (MSE) found in the promoters of many of these genes (6, 40). *NDT80* expression begins during the early stages of meiotic development, where relatively low levels can be detected (7). The initial expression of *NDT80* is reliant on the Ime1/Ume6 transcription factor complex (24, 32). However, maximal expression of *NDT80* occurs during middle sporulation through a positive feed-back mechanism, with Ndt80 activating its own transcription (24). This activity is strongly dependent on the actions of the meiosis-specific protein kinase Ime2 (24, 32).

One of the unique hallmarks of meiotic development is the occurrence of reciprocal recombination between homologous chromosomes (26, 28). The successful completion of meiotic recombination is a prerequisite for the progression from prophase I into the first meiotic chromosomal division (MI). Tight regulation of the machinery driving the transition from prophase I into the meiotic divisions is a conserved theme in gamete development (6, 19, 37). A characteristic rise in Cyclin B-CDK1 activity (Clb-Cdc28 in budding yeast) promotes this transition, and accumulation of this activity occurs only after recombination is complete. MSE-like elements have been identified in the sequences upstream of *CLB1*, *CLB3*, *CLB4*, *CLB5* and *CLB6* (7). These cyclins also display an Ndt80-dependent transcriptional expression during meiotic development. As a testament to Ndt80's role in promoting *CLB* expression during sporulation, *ndt80* mutants are able to complete DNA replication and meiotic recombination, however arrest at the pachytene stage with duplicated but unseparated spindle pole bodies and fully assembled synaptonemal complexes (40). Checkpoint mechanisms during meiotic development, which respond to DNA damage, incomplete DNA replication or a failure in recombination, also result in a meiotic arrest before the chromosome divisions (7, 11, 18, 22, 35). These checkpoints have been proposed to initiate this arrest via the inactivation of Ndt80, thereby preventing the accumulation of Clb-Cdc28 kinase activity, resulting in a pachytene arrest (7, 11, 25).

CLB5 and *CLB6*, expressed in both proliferating and sporulating cells, are the principle cyclins involved in regulating the initiation of DNA replication (8, 29, 35). Though they fulfill this role for both mitotic growth and meiotic development, their activity is absolutely essential for promoting premeiotic S-phase. The start specific transcription factor MBF (MCB-binding factor) regulates *CLB5* expression during mitotic growth by directly associating with MCB elements located in a cluster within the *CLB5* promoter (12). We have shown that MCB-driven expression of *CLB5* during meiotic development is critical for this cyclin to carry out its S-phase function (see Chapter IV).

However, we have determined that the MBF is dispensable for this MCB-mediated transcription (see Chapter III). *CLB5* also contains an MSE-like site within its promoter, and this site has been proposed to promote the Ndt80-dependent expression of this cyclin during the middle stage of sporulation (7). However, results from our promoter analysis in Chapter IV have revealed that this putative MSE may not be a strong activator of Ndt80-mediated transcription. These data have raised questions as to how Ndt80 may be regulating the expression of this cyclin. Ndt80 may be influencing *CLB5* transcription indirectly, through an intermediate upstream regulator. Though no specific examples of this have been uncovered to date, not all middle sporulation genes contain obvious MSE sequences in their promoters, yet these genes are expressed when Ndt80 has initiated the progression from prophase I into the meiotic divisions (6). This strongly favors the possibility of intermediate regulators responding to Ndt80 activation. Another possibility could be that other MSE sequences exist within the *CLB5* promoter that have previously been overlooked. Multiple MSEs have been identified in the promoter of a number of middle sporulation genes such as *NDT80* and *CLB1* (7, 24).

In this study, we confirm that the previously identified MSE within the *CLB5* promoter is not essential to the Ndt80-dependent expression of this cyclin during sporulation. However, Ndt80 specifically binds to the *CLB5* promoter in an *in vivo* assay. Reanalysis of the sequence comprising the *CLB5* promoter has revealed another MSE-like sequence 144 nucleotides upstream from the proposed

MSE. This new MSE proves to be a more favorable binding site for Ndt80 *in vitro*, and has the ability to promote middle sporulation expression of a reporter gene *in vivo*. Also, inactivation of this putative MSE within the *CLB5* promoter dramatically reduces the Ndt80-dependent expression of this B-type cyclin in sporulating cells. Our data strongly implicate this new MSE-like sequence as the primary binding site for Ndt80 within the *CLB5* promoter, and clearly demonstrate that Ndt80 directly regulates this *CLB* cyclin gene.

V.2 – Results and Discussion

V.2.a. – Inactivation of the potential MSE at position -230 does not alter the meiotic expression of *CLB5*: During vegetative growth in *S. cerevisiae*, the B-type cyclin *CLB5* is expressed at peak levels during the G_1/S-phase transition (8, 13, 29). This peak expression is dependent on the start-specific transcription factor MBF (MCB-binding factor) (12, 29). Indeed, a cluster of potential MBF-binding sites, or MCBs (*MluI* Cell cycle Boxes), have been identified within the *CLB5* promoter region (8, 29) (see Chapter IV). In contrast to its expression during proliferative growth, *CLB5* displays a differential pattern of expression in sporulating cells (7) (see Chapter IV). Though *CLB5* expression is induced within two hours following induction of sporulation, peak transcription does not occur until 5 to 8 hrs, coincident with the accumulation of the other B-type cyclins during middle sporulation, and well after its essential role in S-phase. We have previously demonstrated that MCB-directed transcription is crucial for the expression of *CLB5* during premeiotic S-phase, however this expression remains surprisingly independent of MBF activity (see Chapter IV). Also, MCB-mediated expression does not play an important role in the peak transcriptional expression seen during middle sporulation. The meiosis-specific transcription factor Ndt80 is a key regulator of middle sporulation gene expression, and it is this transcription factor that is responsible for the peak *CLB5* expression seen during sporulation (7) (see Chapter III and IV). Consistent with this, a potential MSE situated at position -230 with respect to the *CLB5* start codon has been identified, and is believed to be the binding site for Ndt80 within the *CLB5* promoter (7) (Figure V-1A, see *CLB5* promoter schematic). Work detailed in Chapter IV characterizing the meiotic regulation of *CLB5* offered some insight into the nature of this potential MSE. Specifically, a *CLB5* promoter construct lacking 100bp of sequence upstream of this putative MSE was able to promote an apparently regulated pattern of transcription to a *clb5* reporter gene (see Chapter IV, Δ178 data); *clb5* reporter transcript accumulated coincident with the peak expression of endogenous *CLB5* during middle sporulation. However when compared to the reporter expression from the wild type promoter, the level of expression from this deletion promoter was significantly reduced. This seemed to suggest that this putative MSE could not support the robust *CLB5* expression seen during middle sporulation.

In order to investigate the transcriptional activity of this potential MSE at position -230 (which we will refer to as MSE1), we utilized promoter constructs containing ~1kb of *CLB5* promoter sequence regulating the expression of a *clb5* reporter gene (as in Chapter IV). During sporulation, the wild type promoter construct expressed the *clb5* reporter transcript in a pattern consistent with the endogenous *CLB5* expression (Figure V-1A, *CLB5* wild type). We next analyzed a reporter construct in which MSE1 was mutated (Figure V-1A, see Δ*mse1* promoter schematic). Interestingly, inactivation of MSE1 had no effect on *clb5* reporter gene expression (Figure V-1A, Δ*mse1*). Most notably this Δ*mse1* promoter induces a strong middle sporulation expression of the *clb5* reporter transcripts. To further support these reporter gene results, constructs were created placing a functional *CLB5* open reading frame under the control of a wild type *CLB5* promoter (*CLB5*), or one in which MSE1 was inactivated (Δ*mse1*) (Figure V-1B, see promoter schematics). These constructs were introduced into *clb5 clb6* cells, ensuring the only source of *CLB5* available came from the integrated gene. Again, mutation of MSE1 had no effect on the meiotic *CLB5* mRNA profile (Figure V-1B, compare wild type *CLB5* to Δ*mse1*). *CLB5* still displayed the Ndt80-dependent expression seen at 5 to 8 hrs into sporulation. The fact that the Δ*mse1* promoter still induced peak transcript accumulation during middle sporulation suggests that MSE1 is not essential for the middle sporulation expression of *CLB5*. This implies that MSE1 may not actually be functioning as an efficient binding site for Ndt80, as previously thought. These observations raised questions as to how Ndt80 may be influencing *CLB5* expression. Two possibilities were considered. Could Ndt80 be acting indirectly on *CLB5* transcription, affecting an upstream regulator of this expression? Or, could there be another Ndt80-binding site in the *CLB5* promoter that had previously been overlooked?

V.2.b. – Purified Ndt80 binds specifically to both WT and Δ*mse1* promoter fragments: We have demonstrated that most of the transcriptional regulation imparted upon *CLB5* can be localized to sequences between -222 and -389 within the promoter of this gene, which we have termed the UAS^{CLB5} (see Chapter IV). To investigate whether Ndt80 interacts with this region of *CLB5*, we generated promoter fragments via PCR, encompassing the entire UAS^{CLB5} (Figure V-2A). Table V-1 lists the *CLB5* PCR primers used to amplify the UAS^{CLB5} and their relative positions within the *CLB5* promoter (SAR6/SAR11). Purification of recombinant Ndt80 from *E. coli* was performed using Immobilized-Metal Affinity Chromatography (IMAC). Ndt80 expressed in this way has been shown to display specific DNA-binding activity towards consensus MSE sequences (33). Both WT and Δ*mse1* promoter fragments were ^{32}P-endlabelled and analyzed using electrophoretic mobility shift assays (EMSA) to determine the affinity of purified Ndt80 for each of these regions (Figure V-2B). Lane 1 in each assay represents total added probe (1pmol). Upon addition of purified Ndt80 (~14pmol), a number of shifted species were seen (Figure V-2B, WT and Δ*mse1*). Titration with an increasing amount of poly dI:dC (a non-specific nucleic acid binding competitor) revealed a single shifted species persisting in both assays, demonstrating that Ndt80 displays relatively strong affinity for both of these promoter fragments (Figure V-2B, lanes 2-7 for WT and Δ*mse1*). This suggests a highly specific interaction in both cases, which is revealed by the ability to effectively compete this binding with excess unlabeled specific MSE competitor (Figure V-2B, lanes 8-9 for WT and Δ*mse1*). Interestingly, this specific binding appeared independent of MSE1. Hence, these results demonstrate that Ndt80 can bind specifically to the *CLB5* promoter at a site distinct from this proposed MSE.

V.2.c. – Ndt80 binds specifically to the 5' region of UAS^{CLB5}: To determine where within the UAS^{CLB5} Ndt80 is binding, smaller PCR fragments were created, further dissecting this region of the *CLB5* promoter. These fragments, which we will refer to as Upstream Regulatory Sequences (URS), divide the UAS^{CLB5} in half, but maintain 30bp of overlapping sequence. Figure V-3A defines these promoter fragments, each shown aligned to the relative position they represent within the UAS^{CLB5}; note, $\mathrm{URS}^{\Delta mse1}$ contains the Δ*mse1* mutation. Table V-1 lists the *CLB5* PCR primers used to amplify these promoter fragments and their relative positions within the *CLB5* promoter (SAR6/SAR9 for $\mathrm{URS}^{\mathrm{A}}$; SAR8/SAR11 for $\mathrm{URS}^{\mathrm{MSE1}}$ and $\mathrm{URS}^{\Delta mse1}$). These fragments were ^{32}P-endlabeled and used in electrophoretic mobility shift assay (EMSA) to determine which of these regions displayed strong binding to purified Ndt80 (Figure V-3B). An equivalent amount of probe (1pmol) was added to each respective lane. Lanes 1, 4 and 7 are total probe added for $\mathrm{URS}^{\mathrm{A}}$, $\mathrm{URS}^{\mathrm{MSE1}}$ and $\mathrm{URS}^{\Delta mse1}$ respectively. Upon addition of purified Ndt80 (~14pmol), all probe was bound as evidenced by the shifted species seen and the disappearance of the free unbound probe (Figure V-3B, lanes 2, 5 and 8). Addition of 150X molar excess poly dI:dC revealed a strongly bound species (Figure V-3B, lanes 3, 6 and 9). To examine which of these fragments bound most tightly to Ndt80, we compared the amount of labeled probe remaining unbound in the presence of poly dI:dC. Stronger binding would be represented by less remaining unbound probe. To determine the percentage of unbound probe relative to total probe added, we treated the data as follows: 1) We first calculated the amount of unbound probe in a given lane with respect to the total signal in that lane. 2) Then, to factor out background signals, we determined the percentage of unbound probe relative to the total free probe in the first lane of each fragment set. These percentages are presented below their respective lanes in Figure V-3B (see % Unbound). In the presence of 150X molar excess poly dI:dC, approximately 65% of the URS$^{\mathrm{MSE1}}$ probe remained unbound by Ndt80 (Figure V-3B, lane 6). Inactivation of MSE1 (URS$^{\Delta mse1}$) did lead to a decrease in Ndt80 binding, with 97% of the probe remaining unbound (Figure V-3B, lane 9). This suggests that some specific Ndt80 binding may be occurring at MSE1. Interestingly, in contrast to the relative affinities seen for URS$^{\mathrm{MSE1}}$ and URS$^{\Delta mse1}$, Ndt80 displayed a significantly greater affinity for URS$^{\mathrm{A}}$, with only 28% of the probe remaining unbound in the presence of 150X poly dI:dC (Figure V-3B, lane 3). This would indicate that this region of the *CLB5* promoter may contain a previously overlooked Ndt80 binding site. Therefore, due to the stronger relative binding seen in URS$^{\mathrm{A}}$, and since inactivation of MSE1 does not alter the meiotic expression of *CLB5*, we propose that this 5' region of the UAS^{CLB5} contains a specific Ndt80 binding site, and this site may be the primary mediator of peak *CLB5* expression.

V.2.d. – URS^A contains an MSE-like sequence: By analyzing the promoters of a number of middle sporulation genes, a consensus MSE sequence was determined (5’-gNCRCAAAW-3’; lower case g indicates weak conservation, N indicates a non-conserved position, R represents a purine nucleotide, and W indicates either A or T) (6, 23). However, it was realized that a perfect fit to this putative consensus sequence was not necessarily required for Ndt80 directed transcription. In fact, many middle sporulation genes contained variant MSE sequences (eg. *CLB3* and *CLB4*). Indeed, at the time when this consensus was established, it was understood that MSE sequence requirements for Ndt80 recognition had yet to be determined. Since then, a number of groups have pursued this avenue, revealing surprising complexities to the Ndt80/MSE interaction. Structural analysis of the Ndt80 DNA-binding domain has revealed a central β-sandwich structure characteristic of an s-type Ig fold (9, 16). In fact, Ndt80 represents the first non-metazoan member of the Ig-fold family of transcription factors, which includes members of the p53 (5), Rel/NFκB (4, 10), STAT (1), CBF/Runx (2, 36), and T-Box families (21). When bound to the MSE, a number of interactions occur through a large protein-DNA interface (16). Specific contacts are made between GC-rich regions of the MSE sequence and three arginine residue side chains through the DNA major groove, and specific contacts with the AT-rich region of the MSE are largely made through the DNA minor groove. Ndt80 also appears to make specific associations to the central 5'-pyrimidine-guanine-3' sequence (15). The greater flexibility of these 5'YpG-3' steps allow for unique interactions between a single amino acid side chain and these two consecutive base-pairs. Interestingly, though not essential for its DNA-binding activity, the amino-terminal 40 amino acids of Ndt80 also influence specific interactions with MSE sequences (33). Based on this structural data, mutational analysis of the MSE has reinforced the importance of the 5'-RCAAAW-3' core sequence, conservation of which remains an essential requirement for Ndt80 binding (16, 27).

Based on consensus comparison, it is understandable why MSE1 at position -230 (5'-AACGCAAAT-3') was strongly considered to be the Ndt80 target. This sequence only differs in the weakly conserved G. However, we have reported here that Ndt80 does not seem to display relatively strong binding to this site in an *in vitro* assay (Figure V-3B, URS^{MSE1}). Nor does inactivation of this MSE cause any defect in meiotic expression of *CLB5* (Figure V-1, *Δmse1* promoter). Given that Ndt80 displays stronger binding to *CLB5* promoter fragment URS^{A} in mobility shift assays (Figure V-3B, URS^{A}), we were encouraged to further scrutinize this region in the *CLB5* promoter in an effort to predict where exactly within this sequence Ndt80 may be interacting. Our search revealed a sequence, 5'-GGTACAAAA-3', at position -374 which distinctly resembles an MSE (Figure V-4A). This element maintains the conserved core sequence but contains an obvious variation to the consensus at position 3 (C to T).

In a mutational study by Pierce et al. (2003), a corresponding change made in the *SMK1* MSE proves to cause a significant decrease in Ndt80 binding and transcriptional expression (27). However, results from the same mutational study demonstrated that MSEs are strongly influenced by sequences immediately surrounding this consensus element. With respect to the *SMK1* promoter, significant contributions to binding and transcriptional activity are made by nucleotides 5' to the MSE sequence. This same principle may be true for MSEs regulating other middle sporulation genes. Therefore, nucleotides flanking the putative MSEs within the *CLB5* promoter may be significantly influencing transcriptional activity from these sites. However, based on the in-depth analysis of the *SMK1* MSE sequence (27), an evaluation of the putative *CLB5* MSEs reveals nothing definitive to explain any possible differences between these two *CLB5* MSEs. Since MSEs appear to be influenced by nucleotides surrounding the consensus sequence, these elements may be functioning in a very context-dependent manner. Therefore it is reasonable to consider that different sequence requirements may be employed by different promoters. Also, *SMK1*, which encodes a mitogen-activated protein kinase (MAPK) homolog, is repressed during vegetative growth and early sporulation by the transcriptional repressor Sum1 (27). Sum1 specifically represses a subset of middle sporulation genes by binding to sites that overlaps with the MSE sequence. *CLB5* expression is required during growth and early sporulation, and is an unlikely target of Sum1 repression. This might suggest that MSEs within the *CLB5* promoter may be in a different regulatory environment than the MSE in *SMK1*. Therefore, these *CLB5* MSEs may have different determinants for transcriptional activation by Ndt80.

To determine if this new putative MSE, which we will refer to as MSE2, is a functional Ndt80 binding site within the *CLB5* promoter, another promoter fragment was created in which this site was mutated (Figure V-4B, URS$^{\Delta mse2}$); note, in light of the MSE2 sequence we identified in URS$^{\mathrm{A}}$, we chose to re-designate this fragment as URS$^{\mathrm{MSE2}}$. All four URS fragments were ^{32}P-endlabeled and assayed for Ndt80 binding using EMSAs as before (Figure V-4C). Table V-1 lists the *CLB5* PCR primers used to amplify these promoter fragments (SAR6/SAR9 for URS$^{\mathrm{MSE2}}$ and URS$^{\Delta mse2}$; SAR8/SAR11 for URS$^{\mathrm{MSE1}}$ and URS$^{\Delta mse1}$). Each binding assay contained 1pmol of respective labeled probe. Ndt80 binding to each fragment was challenged with increasing addition of poly dI:dC up to a 150X molar excess (URS$^{\mathrm{MSE2}}$, lanes 2-5; URS$^{\Delta mse2}$, lanes 9-12; URS$^{\mathrm{MSE1}}$, lanes 16-19; URS$^{\Delta mse1}$, lanes 23-26). In all cases, a single shifted species emerged, indicating relatively strong Ndt80 binding (Figure V-4C). Ndt80 displayed considerably higher affinity for URS$^{\mathrm{MSE2}}$ compared to URS$^{\mathrm{MSE1}}$, as evidenced by the greater persistence of the specific shifted species in the URS$^{\mathrm{MSE2}}$ assays with increasing addition of poly dI:dC (Figure V-4C, compare lanes 2-5 to lanes 16-19). Indeed, greater than 50% binding was achieved for URS$^{\mathrm{MSE2}}$ with the addition of 150X poly dI:dC, whereas only 50X poly dI:dC was needed to achieve 50% binding in URS$^{\mathrm{MSE1}}$. Thus, based on these data, it could be argued that URS$^{\mathrm{MSE2}}$ displayed about 3 times the relative affinity for Ndt80 compared to URS$^{\text{MSE1}}$. However, due to the sensitivity of these assays and the minute amounts of substrates involved in each binding reaction, such an absolute comparison may not be entirely prudent for these fragments sets. Nevertheless, a qualitative comparison of the poly dI:dC competition profiles does strongly favor our assertion that URS$^{\text{MSE2}}$ displays greater Ndt80 affinity. Interestingly, mutation of MSE2 produced a very dramatic effect on Ndt80 binding (Figure V-4C, see URS$^{\Delta mse2}$). As with URS$^{\text{MSE1}}$, only 50X excess poly dI:dC was required to produce 50% binding in URS$^{\Delta mse2}$, compared to the 150X excess required for URS$^{\text{MSE2}}$. Therefore, poly dI:dC becomes a much better competitor for this fragment, and relative binding affinity drops approximately 3 fold compared to URS$^{\text{MSE2}}$ containing the wild type MSE2. This data demonstrates that Ndt80 binding to URS$^{\text{MSE2}}$ is dependent on MSE2, implicating this putative MSE as an Ndt80 binding site within the *CLB5* promoter. In contrast to MSE2, mutation of MSE1 did not produce a similar dramatic decrease in Ndt80 affinity (Figure V-4C, see URS$^{\Delta mse2}$). A consistent profile for poly dI:dC competition was seen for URS$^{\text{MSE1}}$ and URS$^{\Delta mse1}$, with both reaching maximal competition at comparable levels. This would suggest that MSE1 may not be functioning as a strong Ndt80 binding site in the *CLB5* promoter. Along with higher affinity, Ndt80 also displays greater specific binding to MSE2. This greater specificity is evident from the increased competition seen by 25X MSE binding competitor compared to 150X poly dI:dC for URS$^{\text{MSE2}}$ (Figure V-4C, compare lane 6 to lane 5). This specific competition was not detected for URS$^{\Delta mse2}$, URS$^{\text{MSE1}}$ and URS$^{\Delta mse1}$. These fragments reached comparable levels of competition under both specific and non-specific conditions tested. This might possibly indicate that a higher degree of non-specific interactions were occurring between Ndt80 and these other fragments - if specific competitors fail to produce an effect, there may not be any specific interactions to compete away. Therefore, MSE2 in *CLB5* promoter fragment URS$^{\mathrm{MSE2}}$ appears to be a more favorable Ndt80 binding site compared to MSE1 in URS$^{\mathrm{MSE1}}$.

V.2.e. – The *CLB5* promoter contains two MSEs with differing affinities for Ndt80: A number of middle-sporulation genes have been shown to contain multiple MSEs within their promoter regions (7). For instance, two MSEs have been identified within the *NDT80* promoter at positions -221 and -86 relative to the start codon (24). *NDT80* displays a prominent increase in transcription during middle sporulation stages, dependent on the presence of these two MSEs and the pre-middle expression of Ndt80. Work done on the expression of truncated *ndt80* mini-gene derived transcripts driven by the *NDT80* promoter has demonstrated that both MSEs within this gene may be functional regulatory sites, and may work together to promote middle sporulation expression of *NDT80* (24). Taking these results into consideration, we questioned whether a similar mechanism may be working in the *CLB5* promoter. Though we demonstrated that Ndt80 has a relatively low affinity for MSE1, any possible contribution to *CLB5* transcription from this MSE should not be ruled out. To this end, we returned to analyzing fragments encompassing the entire UAS^{CLB5}. Along with the wild type (*CLB5*) and Δ*mse1* fragments described in Figure V-2, we generated two new fragments, Δ*mse2* (mutation of MSE2) and Δ*mse1*Δ*mse2* (mutation of both potential MSEs in *CLB5*) (Figure V-5A). These UAS^{CLB5} fragments were compared for Ndt80 binding in the presence of 30X and 100X molar excess poly dI:dC (Figure V-5B). Based on the resulting unbound probe, it is apparent that little difference could be detected in the affinity of Ndt80 for the wild type *CLB5* fragment and the Δ*mse1* fragment (Figure V-5B, compare lane 3 to lane 6). Ndt80 displayed a greater affinity for both these fragments, with approximately 65% of the probe becoming bound by this transcription factor in both reactions. This may suggest that MSE1 does not contribute to any specific Ndt80 binding in this fragment. As expected, deletion of MSE2 decreased the relative affinity of Ndt80 for this UAS^{CLB5} fragment by almost 2-fold (Figure V-5B, compare lane 3 to lane 9). Interestingly, deletion of both MSEs from this UAS^{CLB5} fragment decreased Ndt80 binding to this region by a further 15% (Figure V-5B, compare lane 9 to lane 12). This result agrees with the decrease in Ndt80 affinity seen for $\text{URS}^{\Delta mse1}$ in Figure V-3B. Even though Ndt80 displays a higher affinity for MSE2, these results may implicate MSE1 as a minor contributor to *CLB5* expression. It should be noted that, since MSE1 does match an established MSE consensus, the binding we are observing in this assay may be an effect of this *in vitro* system – Ndt80 may be responding to its inherent affinity for this naked DNA sequence which may be more restricted in the context of chromatin *in vivo*. However, since either scenario cannot be firmly established with the data presented thus far, both possibilities must be considered. Therefore, our data might suggest that *CLB5* is regulated by two MSEs which may be demonstrating unequal transcriptional activities. How might these two regulatory elements be functioning? MSEs in the *CLB5* promoter may be working together to reinforce the robust Ndt80-mediated expression of this cyclin during middle sporulation. In such a scenario, MSE2 could be functioning as the more active site with MSE1 offering a more transient contribution. It may also be possible that these sites are working in a cooperative manner to promote Ndt80-mediated transcription. MSE2, with its greater inherent affinity for Ndt80, may be the initial site targeted by this transcription factor, and this association may promote a more favorable interaction between Ndt80 and MSE1. In contrast, these regulatory elements could be working antagonistically, where Ndt80 binding to the more favorable MSE2 might prevent Ndt80 association with MSE1. This might explain why we only see Ndt80 affinity for MSE1 in the absence of MSE2 in this binding assay (Figure V-5B).

Taken together, our EMSA data demonstrate that Ndt80 binds specifically to the *CLB5* promoter *in vitro*. Though a near-consensus MSE had been previously identified within the *CLB5* promoter (MSE1), our results suggest that this site may not contribute to the majority middle sporulation expression of this B-type cyclin. Instead, a previously overlooked MSE at position -374 from the start codon (MSE2) displays tighter Ndt80 binding.

V.2.f. – MSE2 activates middle sporulation expression of a *lacZ* reporter gene in sporulating cells: Although we demonstrated that Ndt80 can bind MSE2 in an *in vitro* binding assay, this alone does not establish this site as an Ndt80 regulatory element *in vivo*. In order to determine whether this MSE has the ability to promote middle sporulation expression, we developed an *in vivo* reporter gene assay, taking advantage of a *lacZ* expression system - *lacZ* reporter expression has been used successfully by other groups to demonstrate MSE-dependent transcription (7, 27). To analyze the transcription from the putative MSEs in *CLB5*, the very same promoter fragments used in Figure V-4 (URS$^{\text{MSE2}}$, URS$^{\Delta mse2}$, URS$^{\text{MSE1}}$ and URS$^{\Delta mse1}$) were inserted into an episomal *CYC1-lacZ* expression vector and transformed into wild type diploid SK1 cells. These cells were then induced to sporulate and samples were collected every hour up to 12 hours. URS-driven production of *lacZ* transcript was monitored via Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) (Figure V-6). Total RNA was isolated from these samples and residual DNA was removed using DNaseI. 4μg of total RNA was treated with SuperScriptIII reverse-transcriptase (Sigma) and the resulting cDNA was subjected to PCR amplification using primer sets specific for the *lacZ* transcript. Control primer sets specific for *ACT1* and *CLB5* were also included in the PCR reactions as internal loading and sporulation controls respectively. Table V-1 lists the RT-PCR primer-sets used for the initial cDNA 1st strand synthesis (RT primers) and the subsequent amplification of the cDNA product (FWD/RT pairs). Primer-sets were designed to produce RT-PCR products with distinct mobilities on an agarose gel. PCR products were analyzed via Southern blotting technique to visualize relative cDNA abundance (Figure V-6A and -6B, see Southern blots). Blots were probed simultaneously for *lacZ*, *CLB5* and *ACT1*, and radio-labeled signal was quantitated by phosphorimager. The *in vivo lacZ* reporter expression seen from URS^{MSE2} demonstrated a transcriptional profile consistent with middle sporulation gene expression (Figure V-6A, see top graph). An increased accumulation of reporter transcript was detected beginning at 5 hours after induction of sporulation, and this expression pattern coincides well when normalized to the endogenous *CLB5* transcription (Figure V-6A, see bottom graph). A comparison of the reporter expression from URS^{MSE2} versus $\text{URS}^{\Delta mse2}$ revealed a loss of the peak transcript accumulation upon mutation of MSE2 (Figure V-6A, see top graph). The data strongly demonstrate that mutation of MSE2 within this promoter fragment prevents middle sporulation expression of the reporter gene *in vivo*. Since the *lacZ* reporter expression from URS^{MSE2} normalizes precisely to the endogenous expression of *CLB5*, and mutation of MSE2 abolishes the peak reporter expression which coincides with the Ndt80-mediated expression of *CLB5*, this MSE seems to be an authentic Ndt80-regulated cis-acting sequence *in vivo*.

In contrast to the regulated expression seen in URS^{MSE2}, URS^{MSE1} displayed no such accumulation of reporter signal during the sporulation program (Figure V-6B, see top graph). Rather, a constitutive level of expression was observed, which diverged from the profile of endogenous *CLB5* transcription in these cells (Figure V-6B, see bottom graph). This would seem to indicate that this promoter fragment does not induce middle sporulation expression of the reporter gene. Indeed, mutation of MSE1 ($\text{URS}^{\Delta mse1}$) did not alter the reporter expression pattern from this region (Figure V-6B, see top graph). Therefore, MSE1 may not be functioning as an Ndt80-binding site and a transcriptional activator *in vivo*. We did observe a constitutive increase in the level of reporter expression from URS$^{\Delta mse1}$, however the reason for this is unclear. It is doubtful that this result indicates the actions of a repressor, and since this does not hinder our analysis of the Ndt80-mediated expression profile, we have not addressed this effect further.

V.2.g. – MSE2-mediated middle sporulation expression of the *lacZ* reporter is dependent on Ndt80: Ndt80-dependent expression of *CLB*s during middle sporulation has been demonstrated previously (7). Though Ndt80 activity against cis-acting promoter elements in *CLB* genes has not been thoroughly investigated, direct interactions between Ndt80 and upstream MSEs is believed to be the mechanism regulating accumulation of *CLB* mRNA during sporulation. Though our *lacZ* reporter expression results in wild type cells clearly demonstrate that peak middle sporulation expression is induced by MSE2, this data does not conclusively demonstrate that this MSE-driven expression is Ndt80-dependent. Therefore, to determine if the meiotic expression of *lacZ* from MSE2 is dependent on Ndt80, we introduced the *lacZ* reporters driven by URS$^{\mathrm{MSE2}}$ and URS$^{\Delta mse2}$ into diploid *ndt80* cells. These transformed cells were induced to sporulate and samples were collected every hour up to 12 hours. Expression of the *lacZ* reporter genes was monitored via Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) and Southern blotting (Figure V-7A and -7C). To demonstrate the loss of middle sporulation gene expression in *ndt80* cells, endogenous *CLB5* expression was compared between wild type cells carrying the URS$^{\text{MSE2}}$-*lacZ* reporter gene and *ndt80* cells carrying either the URS$^{\text{MSE2}}$-*lacZ* reporter (Figure V-7B, top graph) or the URS$^{\Delta mse2}$-*lacZ* reporter (Figure V-7D, top graph). Indeed, the absence of peak middle sporulation expression of endogenous *CLB5* in sporulating *ndt80* mutants confirmed the absence of Ndt80-dependent transcription in these cells. We next analyzed the *lacZ* reporter expression from URS$^{\text{MSE2}}$ and URS$^{\Delta mse2}$ in these *ndt80* mutants. When normalized to endogenous *CLB5* accumulation, *lacZ* reporter expression from both URS$^{\text{MSE2}}$ (Figure V-7B, middle graph) and URS$^{\Delta mse2}$ (Figure V-7D, middle graph) displayed a similar constitutive pattern of expression. Importantly, URS$^{\text{MSE2}}$ no longer induced the middle sporulation expression of its reporter gene in *ndt80* mutants. This was clearly seen when URS$^{\text{MSE2}}$-*lacZ* reporter expression in *ndt80* cells was normalized to the corresponding URS$^{\text{MSE2}}$-*lacZ* reporter expression in wild type cells (Figure V-7B, bottom graph). This altered expression profile clearly demonstrates that expression from URS$^{\text{MSE2}}$ is strongly dependent on Ndt80. This result, combined with the fact that mutation of MSE2 abolishes the peak accumulation of reporter gene transcripts in wild type cells (Figure V-6A), clearly indicates that the Ndt80-dependent expression from URS$^{\text{MSE2}}$ absolutely requires MSE2. Therefore, results from the *lacZ* reporter gene expression support the implication that MSE2 drives middle sporulation expression of *CLB5* and this site is directly targeted by Ndt80 activity.

V.2.h. – Inactivation of MSE2, but not MSE1, reduces the middle sporulation expression of *CLB5*: *in vivo* reporter assays described above strongly implicate MSE2 as a legitimate Ndt80-targeted regulatory element. However, does this site really act as an MSE within the *CLB5* promoter itself? To determine whether this sequence regulates the Ndt80-dependent accumulation of this B-type cyclin during sporulation, more *CLB5* promoter constructs were created. In addition to the wild type (*CLB5*) and Δ*mse1* constructs described in Figure V-1, promoters containing a single inactivation of MSE2 (Δ*mse2*), or a double knockout of both MSE1 and MSE2 (Δ*mse1*Δ*mse2*) were created and placed upstream of a wild-type *CLB5* open reading frame. These constructs were introduced into *clb5 clb6* SK1 mutants, thereby being the sole source of *CLB5* produced in these cells. As in Figure V-1, these cells were induced to sporulate and samples were collected hourly up to 12 hours. RNA isolated from these samples was subjected to Northern blot analysis, where each set was probed for *CLB5* (Figure V-8A). Induction of middle sporulation was monitored by detection of the *NDT80* and *SPS1* transcripts, and loading was controlled for by probing *ACT1*. *CLB5* transcript expression was quantitated by phosphorimager and presented relative to *ACT1* signal (Figure V-8A, see graph). As demonstrated in Figure V-1, mutation of MSE1 did not alter the normal meiotic expression of *CLB5*. Both *CLB5* and Δ*mse1* promoters displayed a prominent peak expression between 5 to 8 hrs post meiotic induction, coincident with *NDT80* and *SPS1* transcription (Figure V-8A, compare wild type *CLB5* to Δ*mse1*). Consistent with the *lacZ* reporter data reported above, inactivation of MSE2 within this promoter diminished the peak middle sporulation accumulation of *CLB5*, instead producing a constitutive pattern of expression (Figure V-8, see Δ*mse2*). This constitutive expression was consistent with that observed for the *CLB5* transcript in sporulating *ndt80* diploids (see Chapter IV), and strongly suggests a loss of Ndt80-driven expression of *CLB5* is observed upon mutation of MSE2. Considering the specific binding Ndt80 demonstrates to this site *in vitro*, and its ability to activate middle-sporulation expression *in vivo*, this site has proven to be the MSE largely responsible for the Ndt80-dependent expression of *CLB5* expression observed during middle sporulation. No added defects in meiotic-*CLB5* expression were apparent from the Δ*mse1*Δ*mse2* promoter (Figure V-8A, see Δ*mse1*Δ*mse2*). Interestingly, though marginal Ndt80-affinity was detected for MSE1, these data suggest that no significant contribution is made towards the middle sporulation expression of *CLB5* by this putative MSE.

Clb5-associated kinase activity is absolutely essential for the successful completion of pre-meiotic DNA replication during sporulation (35). Clb5 has also been implicated to play an important role in spindle assembly and orientation during mitotic growth (30, 31). Clb5's role during MI and MII has not yet been investigated, however it may be appropriate to suggest that this cyclin could be involved in a meiotic process analogous to its proposed role in spindle assembly during mitosis. With such a dramatic decrease in *CLB5* expression during MI and MII in the Δ*mse2* and Δ*mse1*Δ*mse2* strains, we chose to analyze the proportion of cells progressing through MII for each of the constructs described above (Figure

V-7B). No profound differences were seen for the progression of these cells through meiosis. Regardless of which promoter construct was regulating *CLB5* expression, all strains initiated the meiotic divisions in a timely manner, and achieved a relatively high sporulation efficiency. However, a decrease in sporulation efficiency was observed for those cells carrying promoter constructs with Δ*mse2* mutations. For example, cultures expressing *CLB5* from the Δ*mse2* promoter demonstrated a sporulation efficiency of 62.67%, whereas cells expressing *CLB5* from the wild type promoter achieved a sporulation efficiency of 76.67% (these percentages are based on counting 200 cells in three different fields for each strain). This analysis suggests that the reduction in sporulation efficiency seen in cells expressing *CLB5* from the Δ*mse2* promoter may be an authentic defect. Even though the absolute defect in sporulation is minor, this data may demonstrate a role for Clb5 activity during the meiotic nuclear divisions. It may be reasonable to propose that all the Clb cyclins expressed during middle sporulation may be combining their efforts to promote efficient meiotic nuclear divisions (MI and MII). Therefore, a decrease of Clb5 activity in sporulating Δ*mse2* cells may be directly causing the reduced sporulation efficiency observed. Interestingly, such a role for *CLB5* has not been demonstrated previously.

V.2.i. – Conclusion: The meiotic expression of *CLB* genes in *Saccharomyces cerevisiae* occurs in a coordinated manner during middle sporulation, and is strongly dependent on Ndt80 activity. Our analysis of the *CLB5* promoter has clearly demonstrated that Ndt80 directly binds to an MSE sequence within the

UAS^{CLB5} and this association drives the robust peak accumulation of *CLB5* during meiosis. We have identified MSE2 as the primary site targeted by Ndt80 within the *CLB5* promoter. In contrast, MSE1 may not be strongly influencing this expression. The expression of cyclin B, and subsequent activation of CDK1 (MPF) during oocyte maturation in metazoans is an absolute requirement for promoting the transition from prophase to metaphase I (3, 19). Even though no Ndt80 homologs have been discovered in higher eukaryotes, a potentially analogous transcription factor regulating the prophase-meiosis I transition has been described in *Drosophila*. The *always early* (*aly*) gene in *Drosophila* plays a key developmental role in spermatogenesis (17, 39). Most notably, *aly* is required for the meiotic expression of *cyclin B*. Analogous to *ndt80* mutants, germ cells in *aly* mutant testis are unable to accumulate transcripts required for the meiotic nuclear divisions and spermatid differentiation, preventing these cells from progressing through spermatogenesis (38). However, unlike Ndt80, nuclear localization of the Aly protein appears to be an important regulatory mechanism controlling its activity. In early primary spermatocytes, the Aly protein is both cytoplasmic and nuclear. However, in maturing primary spermatocytes, Aly becomes completely nuclear where it associates with chromatin, promoting transcription and influencing overall chromatin structure. Interestingly, the *aly* gene family is conserved from plants to humans (38). However, a role for these genes during spermatogenesis in other organisms has not been described.

V.3-References

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Table V-1
Primers used for creation of *CLB5* promoter fragments and RT-PCR

	Primer	Fwd/Rev*	Position§	Sequence
CLB5 Promoter	SAR6	F	-418	5'-GCAGCGACTAGTCCAGCAAAAGAACGCGCATC-3'
	SAR9	R	-291	5'-GCGGCAGTCGACTTAGCACAGATATGCTGTGG-3‘
	SAR8	F	-320	5'-GCAGCGACTAGTGGGATAGCGCCCACAGCATA-3‘
	SAR11	R	-191	5'-GCGGCAGTCGACGAATTAGTTCCAAGTAGCTT-3‘
CLB5 RT-PCR	DS202	F	+187	5'-CAGCAGGTTCAGGATTCTAAACCAGTGAACAATAATCCT-3‘
	DS180	R	+770	5'-CCTCCTCAAATTTTGCCGCG-3‘
lacZ RT-PCR	LACZFWD	F	+641	5'-CGTTGCTGCATAAACCGACT-3‘
	LACZRT	R	+1100	5'-ACCTGACCATGCAGAGGATG-3‘
ACT1 RT-PCR	ACT1FWD	F	+600	5'-CCAGAAGAACACCCTGTTCT-3‘
	ACT1RT	R	+800	5'-TGGAACGACGTGAGTAACAC-3'

* Fwd/Rev indicates primer orientation relative to direction of the respective promoter and open reading frame.

§ Position is indicated in base-pairs relative to initiation codon of the respective open reading frame.

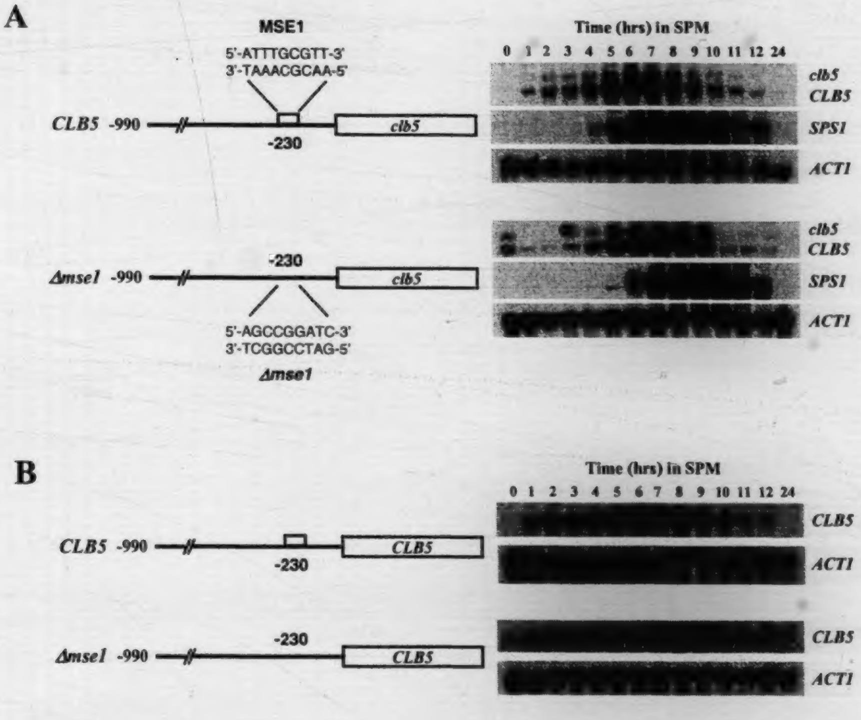

A
MSE1
5'-ATTTGCGTT-3'
3'-TAAACGCAA-5'
CLB5 -990
-230
clb5
Time (hrs) in SPM
0 1 2 3 4 5 6 7 8 9 10 11 12 24
clb5
CLB5
SPS1
ACT1
Δmse1 -990
-230
clb5
5'-AGCCGGATC-3'
3'-TCGGCCTAG-5'
Δmse1
clb5
CLB5
SPS1
ACT1
B
Time (hrs) in SPM
0 1 2 3 4 5 6 7 8 9 10 11 12 24
CLB5 -990
-230
CLB5
CLB5
ACT1
Δmse1 -990
-230
CLB5
CLB5
ACT1

Figure V-1: Mutation of MSE1 (-230) does not alter the Ndt80-dependent expression of *CLB5* during meiotic development. (A) Wild type homozygous diploids carrying *clb5* reporter constructs were induced to sporulate, and RNA samples collected at various time-points were analyzed by Northern Blot for *CLB5, SPS1,2* and *ACT1* mRNA. The reporter transcript (*clb5*) migrates more slowly than the endogenous *CLB5* mRNA (*CLB5*). Promoter schematics for wild type and mutant *Δmse1* reporter constructs are presented (left) corresponding to their respective Northern blot data (right). Relevant promoter details are shown, most notably sequences of the wild type MSE1 and the inactivated sequence Δ*mse1*, and the position of this sequence element relative to the start codon of *CLB5*. Note, wild type reporter data shown was presented in Chapter IV. (B) *clb5 clb6* homozygous diploid cells expressing *CLB5* from either a wild type promoter or the Δ*mse1* promoter were induced to sporulate. RNA samples were collected at time points indicated and subjected to Northern blot analysis. Wild type and Δ*mse1* promoter schematics are shown (left) corresponding to the respective RNA expression profile (right). Northern blots were probed for *CLB5* expression from these promoter constructs and *ACT1* for a loading control. Note, wild type promoter data shown was presented in Chapter IV.

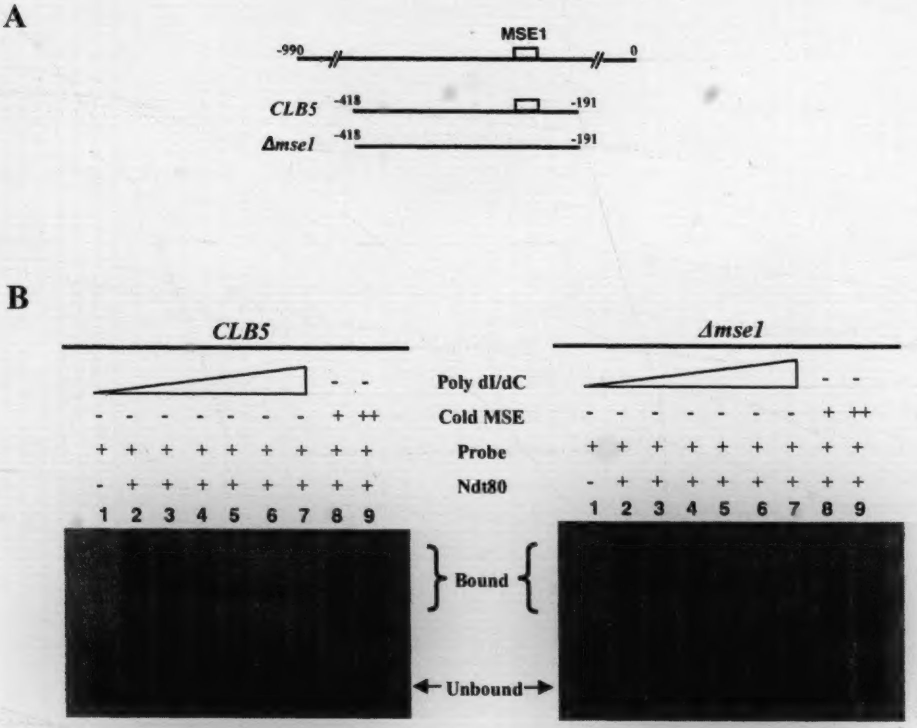
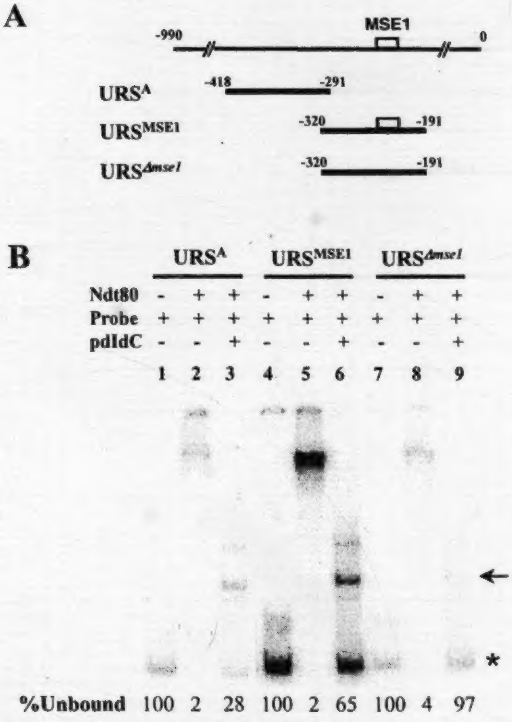


Figure V-2: Recombinant Ndt80 purified by IMAC displays similar relative affinity for both wild type and Δ*mse1* UAS^{CLB5} fragments. (A) UAS^{CLB5} promoter fragments representing wild type *CLB5* and *Δmse1* promoter constructs were produced by PCR. Relative positions of these promoter fragments corresponding to the UAS^{CLB5} are shown, as well as the position of MSE1. (B) These UAS^{CLB5} promoter fragments were ^{32}P-endlabeled and added to binding reactions (1pmol/reaction) with purified Ndt80 (~14pmol/reaction), along with increasing amounts of non-specific binding competitor poly dI:dC and specific MSE duplex binding competitor as indicated. DNA binding was analyzed using native gel mobility shift assays. Free and bound fragments are indicated for each assay. The amounts of poly dI:dC added to lanes 1 through 7 for each gel shift assay are 0µg, 0.002µg, 0.1µg, 0.2µg, 1µg, 2µg, and 5µg respectively. These amounts correspond to 0-, 2-, 10-, 20-, 100-, 200- and 500-fold excess of labeled UAS^{CLB5} promoter fragment. The relative amounts of specific MSE competitor added to lanes 8 and 9 for each assay are 25- and 50-fold excess respectively.



A
-990
MSE1
0
URS^{A}
-418
-291
URS^{MSE1}
-320
-191
URS$^{\Delta mse1}$
-320
-191
B
URS^{A}
URS^{MSE1}
URS$^{\Delta mse1}$
Ndt80 - + + - + + - + +
Probe + + + + + + + + +
pdIdC - - + - - + - - +
1 2 3 4 5 6 7 8 9
*
%Unbound 100 2 28 100 2 65 100 4 97

Figure V-3: Recombinant Ndt80 demonstrates greater binding affinity for URS^{A}. (A) *CLB5* promoter fragments URS^{A}, URS^{MSE1} and URS$^{\Delta mse1}$ were produced by PCR and are shown relative to their corresponding positions in UAS^{CLB5}. The positions that each fragment represents within the *CLB5* promoter is indicated relative to the initiation codon. Note, URS$^{\Delta mse1}$ contains the mutant sequence Δ*mse1*. (B) ^{32}P-labeled URS^{A}, URS^{MSE1} and URS$^{\Delta mse1}$ were assayed for Ndt80 binding affinity using gel shift assay. 1pmol of URS^{A} (lanes 1-3), URS^{MSE1} (lanes 4-6) and URS$^{\Delta mse1}$ (lanes 7-9) were used in each respective binding reaction. Total free fragment added is shown in the first lane for each fragment set indicated (lanes 1, 4, 7). ~14pmol of purified Ndt80 was added to reactions represented by lanes 2-3 (URS^{A}), lanes 5-6 (URS^{MSE1}), and lanes 8-9 (URS$^{\Delta mse1}$). 150-fold excess non-specific poly dI:dC competitor was used for each fragment in lanes 3, 6 and 9. A strongly bound shifted species which persists upon addition of poly dI:dC is indicated by the arrow. Unbound promoter fragment for each lane is indicated by an asterisk. Quantitation of unbound probe is indicated below the panel as percent of total signal in each respective lane, then relative to the total free probe in the first lane of each fragment set.

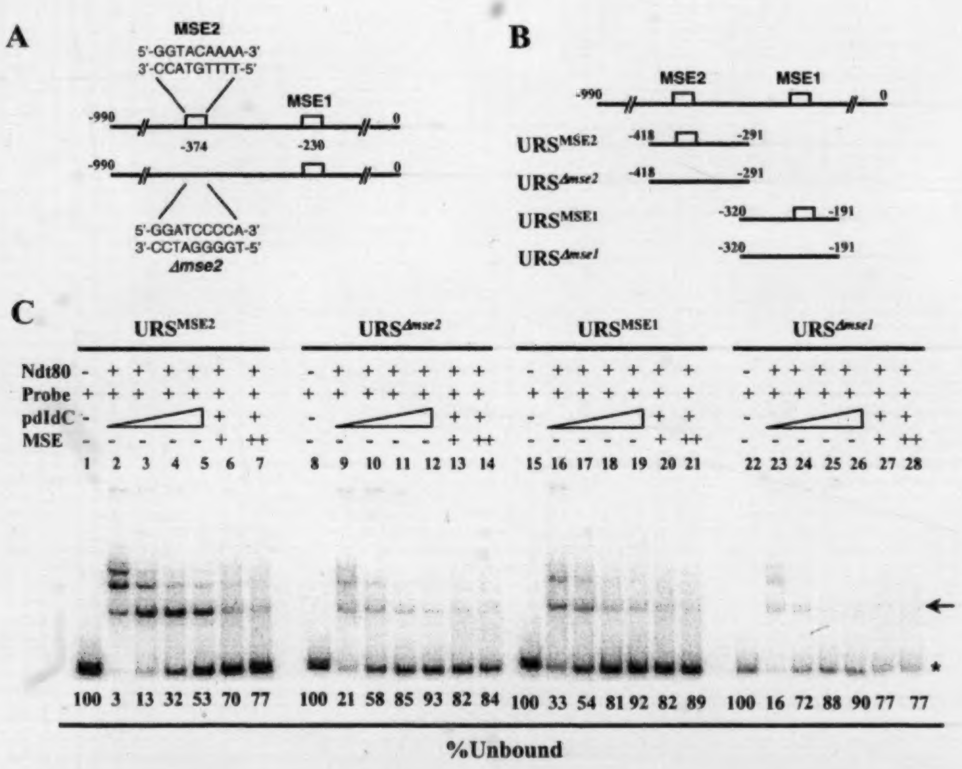

A
MSE2
5'-GGTACAAAA-3'
3'-CCATGTTTT-5'
MSE1
-990
-374
-230
0
5'-GGATCCCCA-3'
3'-CCTAGGGGT-5'
Δmse2
B
MSE2
MSE1
-990
0
URS^MSE2
URS^Δmse2
URS^MSE1
URS^Δmse1
-418
-291
-320
-191
C
URS^MSE2
URS^Δmse2
URS^MSE1
URS^Δmse1
Ndt80
Probe
pdIdC
MSE
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28
100 3 13 32 53 70 77 100 21 58 85 93 82 84 100 33 54 81 92 82 89 100 16 72 88 90 77 77
%Unbound

Figure V-4: Inactivation of MSE2 produces a greater defect in relative Ndt80-binding affinity compared to inactivation of MSE1. (A) Promoter schematics representing the UAS^{CLB5} are shown, presenting both the wild type region and one in which MSE2 is mutated (Δ*mse2*). Note, sequences for MSE2 and Δ*mse2* are indicated, as is the position of this site relative to the start codon. (B) *CLB5* promoter fragments produced by PCR are shown relative to their corresponding positions in UAS^{CLB5}, including URS$^{\Delta mse2}$ which incorporates the Δ*mse2* mutated sequence. (C) URS^{MSE2}, URS$^{\Delta mse2}$, URS^{MSE1} and URS$^{\Delta mse1}$ were ^{32}P-endlabeled and used in binding assays (1pmol) with Ndt80 (~14pmol) to determine the relative affinity that Ndt80 demonstrates for each fragment (URS^{MSE2}, lanes 1-7; URS$^{\Delta mse2}$, lanes 8-14; URS^{MSE1}, lanes 16-21; URS$^{\Delta mse1}$, lanes 22-28). Total free probe is indicated in the first lane of each assay shown (lanes 1, 8, 15, 22) Ndt80/fragment complexes are challenged with increasing amounts of poly dI:dC (URS^{MSE2}, lanes 2-5; URS$^{\Delta mse2}$, lanes 9-12; URS^{MSE1}, lanes 16-19; URS$^{\Delta mse1}$, lanes 23-26). Amounts of poly dI:dC added correspond to 30-, 50-, 100- and 150-fold excess with respect to the total labeled probe. Specific MSE competitor was added in 25- and 50-fold excess in the presence of 30x poly dI:dC (URS^{MSE2}, lanes 6-7; URS$^{\Delta mse2}$, lanes 13-14; URS^{MSE1}, lanes 20-21; URS$^{\Delta mse1}$, lanes 27-28). A strongly bound shifted species which persists with increasing poly dI:dC is indicated by the arrow. Unbound promoter fragment for each lane is indicated by an asterisk. Quantitation of unbound fragments is indicated below the panel as percent of total signal is each respective lane, then relative to the total free probe signal in the first lane of each fragment set.

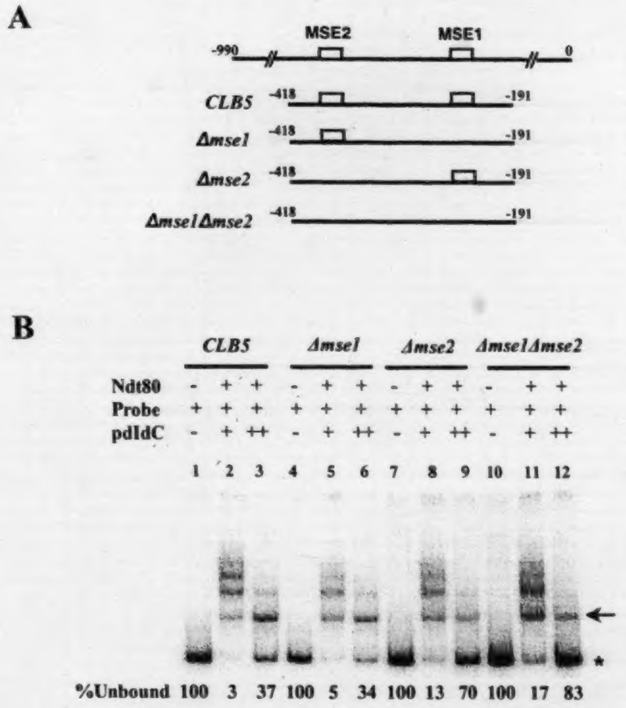

A
MSE2
MSE1
-990
0
CLB5
Δmse1
Δmse2
Δmse1Δmse2
-418
-191
B
CLB5
Δmse1
Δmse2
Δmse1Δmse2
Ndt80 - + + - + + - + + - + +
Probe + + + + + + + + + + + +
pdIdC - + ++ - + ++ - + ++ - + ++
1 2 3 4 5 6 7 8 9 10 11 12
←
*
%Unbound 100 3 37 100 5 34 100 13 70 100 17 83

Figure V-5: Inactivation of MSE2 significantly reduces Ndt80-binding affinity to UAS^{CLB5} fragments. (A) UAS^{CLB5} promoter fragments representing wild type *CLB5*, Δ*mse1*, Δ*mse2* and Δ*mse1* Δ*mse2* promoter constructs were produced by PCR. Relative positions of these promoter fragments corresponding to the UAS^{CLB5} are shown, as well as relevant features of each fragment. (B) UAS^{CLB5} promoter fragments were ^{32}P-endlabeled and used in Ndt80 binding assays (wild type *CLB5*, lanes 1-3; Δ*mse1*, lanes 4-6; Δ*mse2*, lanes 7-9; Δ*mse1* Δ*mse2*, lanes 10-12). Total free fragment added to each assay (1pmol) is shown in the first lane of each set (lanes 1, 4, 7, 10). ~14pmol of purified Ndt80 was added to consecutive binding reactions for each fragment set indicated in the presence of 30- and 100-fold excess poly dI:dC respectively (wild type *CLB5*, lanes 2-3; Δ*mse1*, lanes 5-6; Δ*mse2*, lanes 8-9; Δ*mse1* Δ*mse2*, lanes 11-12). A strongly bound shifted species which persists upon addition of poly dI:dC is indicated by the arrow. Unbound promoter fragment for each lane is indicated by an asterisk. Quantitation of unbound fragments is indicated below the panel as percent of total signal in each respective lane, then relative to the total free probe signal in the first lane of each fragment set.

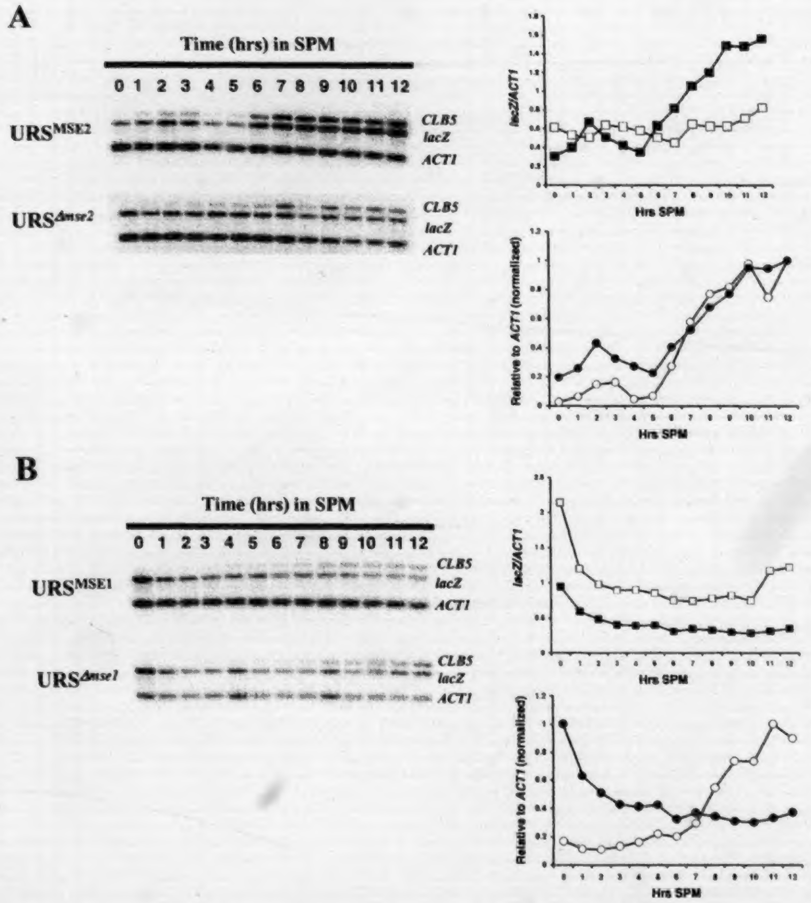


Figure V-6: MSE2, but not MSE1, can drive middle sporulation expression of a *lacZ* reporter gene. (A) Southern blot analyses of RT-PCR samples from sporulating cells expressing the *lacZ* reporter from URS^{MSE2} and URS$^{\Delta mse2}$ are shown; blots were probed for *CLB5*, *ACT1* and *lacZ*. Top graph - Meiotic expression of *lacZ* relative to *ACT1* from URS^{MSE2} (filled squares) and URS$^{\Delta mse2}$ (open squares) are compared. Bottom graph - *lacZ* expression (filled circles) from URS^{MSE2} is normalized to endogenous *CLB5* expression (open circles) throughout meiotic development. (B) Southern blot analyses of RT-PCR samples from sporulating cells expressing the *lacZ* reporter from URS^{MSE1} and URS$^{\Delta mse1}$ are shown; blots were probed for *CLB5*, *ACT1* and *lacZ*. Top graph - Meiotic expression of *lacZ* relative to *ACT1* from URS^{MSE1} (filled squares) and URS$^{\Delta mse1}$ (open squares) are compared. Bottom graph - *lacZ* expressed from URS^{MSE1} (filled circles) is normalized to endogenous *CLB5* expression (open circles) throughout meiotic development.

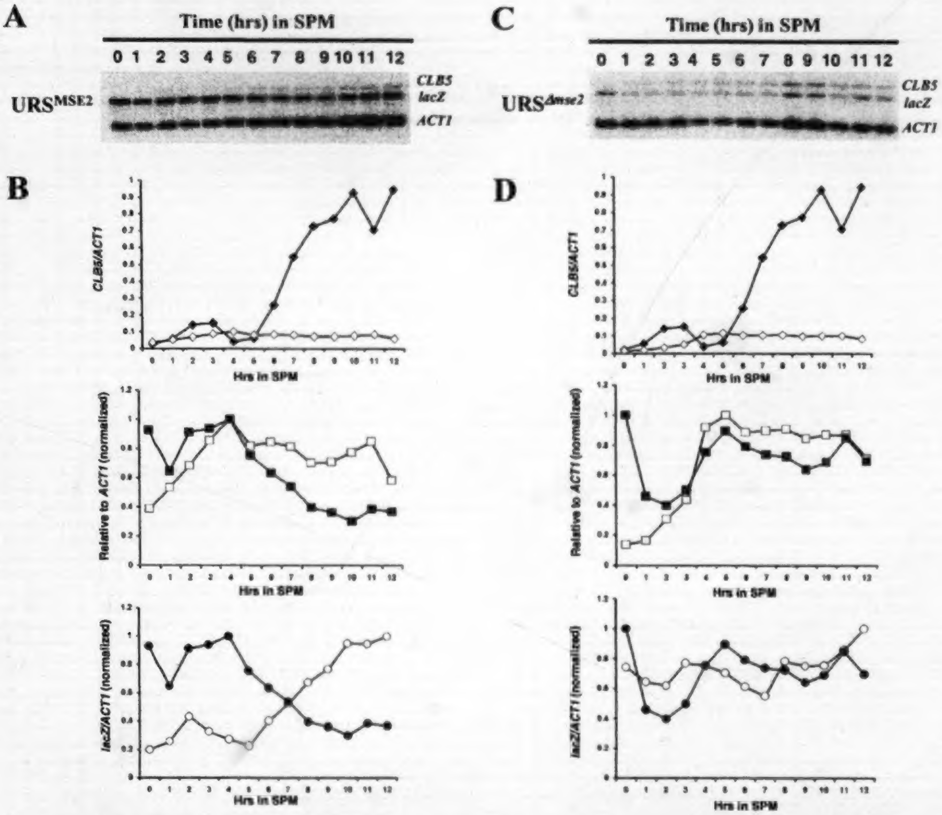


Figure V-7: Middle sporulation expression of *lacZ* regulated by MSE2 is dependent on Ndt80. (A) Southern blot analysis of RT-PCR samples from sporulating *ndt80* cells expressing the *lacZ* reporter from URS^{MSE2} is shown; blots were probed for *CLB5*, *lacZ* and *ACT1*. (B) Top graph – Endogenous meiotic *CLB5* expression from wild type cells harboring the URS^{MSE2}-*lacZ* reporter from Figure V-6A (filled diamonds) is compared to endogenous meiotic *CLB5* expression from *ndt80* cells harboring the same reporter (open diamonds). Middle graph - *lacZ* expression from URS^{MSE2} in *ndt80* cells (filled squares) is normalized to endogenous *CLB5* expression (open squares) throughout meiotic development. Bottom graph - *lacZ* expression from URS^{MSE2} in *ndt80* cells (filled circles) is normalized to *lacZ* expression from URS^{MSE2} in wild type cells (open circles) throughout meiotic development. (C) Southern blot analysis of RT-PCR samples from sporulating *ndt80* cells expressing the *lacZ* reporter from URS$^{\Delta mse2}$ is shown; blots were probed for *CLB5*, *lacZ* and *ACT1*. (D) Top graph - Endogenous *CLB5* expression from wild type cells harboring the URS$^{\Delta mse2}$-*lacZ* reporter from Figure V-6A (filled diamonds) is compared to endogenous *CLB5* expression from *ndt80* cells harboring the same reporter (open diamonds). Middle graph - *lacZ* expression from URS$^{\Delta mse2}$ in *ndt80* cells (filled squares) is normalized to endogenous *CLB5* expression (open squares) throughout meiotic development. Bottom graph – *lacZ* expression from URS$^{\Delta mse2}$ in *ndt80* cells (filled circles) is normalized to *lacZ* expression from URS$^{\Delta mse2}$ in wild type cells (open circles) throughout meiotic development.

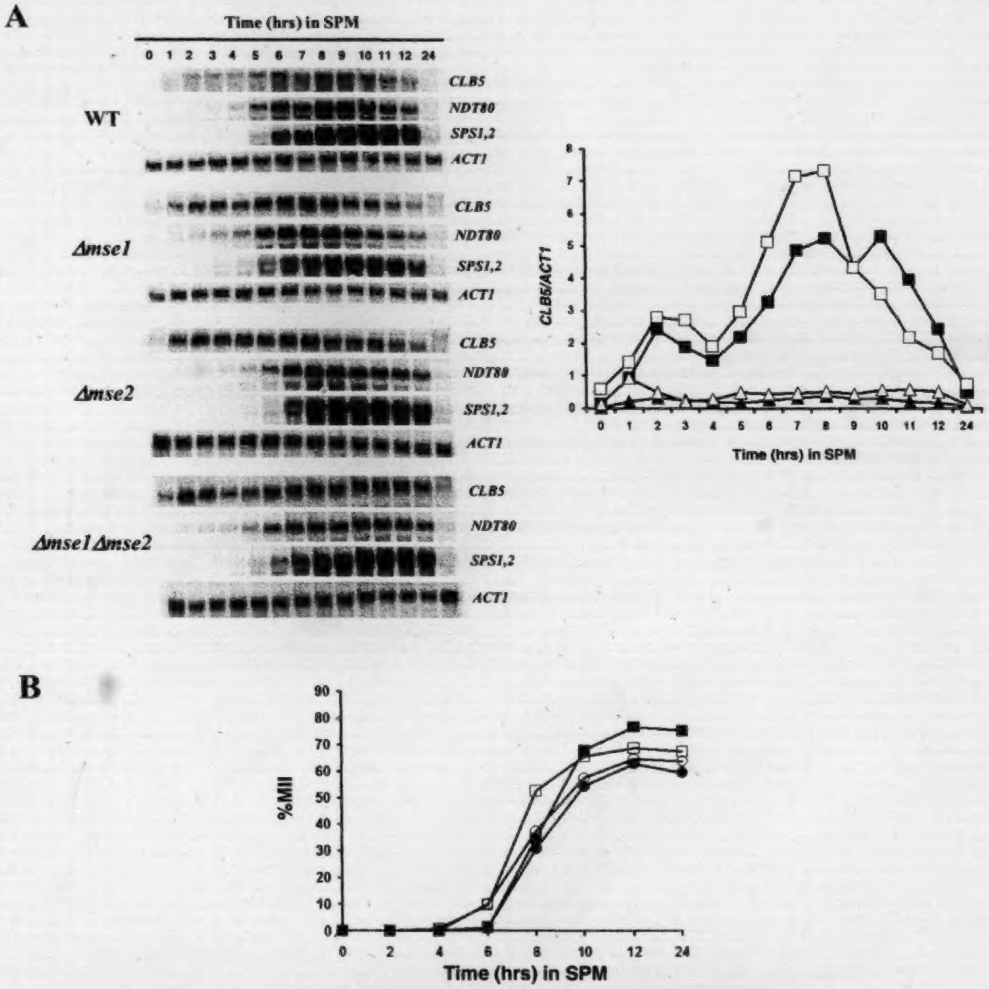


Figure V-8: Middle sporulation expression of *CLB5* is dependent on MSE2 (-374). *clb5 clb6* homozygous diploid cells expressing *CLB5* from the wild type *CLB5*, Δ*mse1*, Δ*mse2* and Δ*mse1*Δ*mse2* promoters were induced to enter sporulation. (A) RNA samples were collected at time points indicated and subjected to Northern blot analysis. Northern blots were probed for *CLB5*, *NDT80*, *SPS1,2* and *ACT1* as indicated. *CLB5* expression from the WT promoter construct (filled squares), Δ*mse1* promoter (open squares), Δ*mse2* promoter (filled triangles), and Δ*mse1*Δ*mse2* (open triangles) was quantitated and is presented relative to the *ACT1* loading control (graph at right). (B) Chromosomal segregation was monitored from sporulating samples of cells expressing *CLB5* from the WT promoter construct (filled squares), Δ*mse1* promoter (open squares), Δ*mse2* promoter (filled circles), and Δ*mse1*Δ*mse2* promoter (open circles) via DAPI staining of nuclei. Percentage of cells completing MII is presented.

Chapter VI

Conclusion:

Reflection and Recommendations

VI.1 – A Graduate Thesis in 176bp of DNA

Inquisitiveness and ingenuity have been characteristic traits of the human race since the dawn of our evolution. For all the advances made in all the four corners of the world, one fundamental truth underlies our quest for knowledge; no question investigated has ever become fully realized. Even with the rise of the modern scientific method, which has spawned an exponential increase in our depth of understanding, only one fully conclusive result emerges – there is still much more to learn. With this in mind, I evaluate my presentation of the meiotic regulation of the S-phase cyclin *CLB5* in *Saccharomyces cerevisiae*, and reflect back on my years of study in this field.

When I began my investigation, I admit that I did not fully appreciate the intricacies of eukaryotic transcriptional regulation, nor did I comprehend the difficulties inherent in the strategies employed towards study of this fundamental biological process. However, my inexperience in the matter was soon challenged. Failures are hardly unheard of in scientific research, and, in fact, most would agree that persistence through such road-blocks builds the foundation for scientific character and integrity. Indeed, if I were permitted, I would reveal that I encountered many more pot-holes at my lab bench than I did driving the streets of Edmonton during the spring-time. Whether spawned from errors of my own, or from forces beyond my control, these uphill battles in my work produced a great deal of frustration. However, as I review my thesis in its entirety, I cannot help but feel pleased about my overall accomplishment. Within 176 base-pairs of

DNA sequence, I have uncovered a significant amount of information - enough to produce an entire graduate thesis. My effort over the years has brought me to this stage in my career, though I must acknowledge and praise my supervisor and mentor, Dr. Dave Stuart, for his intuitive foresight regarding the direction of my research - his faith in me and enthusiasm for my project encouraged me to push forward. As a result, the large body of data I have collected, and the convincing nature of much of my work has allowed me to make solid contributions to the field of cyclin research. Of course, these steps are still only the beginning towards understanding how an essential S-phase cyclin is expressed during meiotic development. However, the doors which I have opened with my work illuminate some intriguing avenues of investigation.

VI.2 – *CLB5*: A Differentially Regulated Gene in *S. cerevisiae*

Throughout the course of my work on this project, my principle goal has always been to discover why the expression of the S-phase cyclin *CLB5* is absolutely required for the efficient induction of meiotic DNA replication. We reasoned that a possible difference in the regulation of *CLB5* expression between mitotic proliferation and meiotic development may contribute to the essential requirement for this cyclin during premeiotic S-phase. Indeed, we have discovered significant differences in the regulation of this cyclin during these two processes. However, we must note that we have not uncovered any definite answers to undeniably explain the essential requirement for *CLB5* during meiotic development. Nevertheless, our extensive analysis of the *CLB5* promoter has produced some intriguing results, which has allowed us to reason why this differential requirement for *CLB5* exists.

VI.2.a. – The Duality of MBF: Through our characterization of meiotic *CLB5* expression in various mutant strains, we unexpectedly discovered that the MBF does not regulate this cyclin during sporulation. This came in striking contrast to well-established experiments in proliferating cells demonstrating that periodic expression of *CLB5* is dependent on Mbp1. Interestingly, we were also able to establish that Mbp1 is indeed a functional transcription factor during meiotic development. Therefore, this suggested that the MBF appears to regulate only a subset of its known target genes during sporulation. To date, very little is known about MBF regulation and transcriptional activity. Genetic data investigating the interactions between MBF, and the related transcription factor SBF, have strongly indicated that the mechanisms employed by these factors are considerably complicated (see Chapter III). Based on our observations we have proposed that the MBF may be susceptible to meiosis-specific regulatory mechanisms, thereby producing the differential transcriptional activity we report during sporulation. A number of conceivable strategies could be proposed to support this regulation. The most direct explanation could involve unique post-translational modifications of Mbp1 that could alter its specificity for its DNA binding site, the MCB element. This type of mechanism would rely on subtle differences in MCB sequences that may equate to larger differences in Mbp1 affinity. Therefore, this strategy could potentially illuminate subclasses of MCB sites. To our knowledge, Mbp1 has not been shown to be phosphorylated *in vivo*, and our analysis of meiotic Mbp1 protein accumulation does not suggest any changes in the state of this protein throughout sporulation (Chapter III). We recognize that this observation is by no means conclusive, and so post-translational modification still remains a possibility. Meiosis-specific MBF-associated proteins may also be influencing Mbp1 activity during meiotic development. Different associated factors may alter MBF affinity towards certain gene promoters. These factors may be directly influencing MBF binding or may be involved in recruiting MBF to specific targets. Also, these factors may be responding to meiosis-specific regulations, supporting the distinct meiotic MBF activity. Another possible mechanism responsible for the unique meiotic regulation of *CLB5* may involve other potential forms of MBF, for example meiotic variants of this transcription factor. Recent discoveries in *Schizosaccharomyces pombe* concerning meiotic variants of DSC1 have allowed us to propose this analogous model for the MBF in *S. cerevisiae* (Chapter III). Not only does this support the possibility that meiosis-specific factors are involved in MBF activity, but this comparison also proposes that novel MCB-binding factors may exist in sporulating cells. This is a possibility which we have been able to address, albeit only in a preliminary manner (see below).

VI.2.b. – Reprogramming *CLB5* for Meiotic Development: To determine why *CLB5* is essential for premeiotic S-phase but not for mitotic DNA replication, we considered the possibility that the expression of this cyclin may be regulated differently during sporulation. Indeed, *CLB5* is expressed *de novo* upon meiotic induction, confirming that a mechanism functions during sporulation to promote this expression (Chapter IV). Through an in-depth promoter analysis, we narrowed down this regulation to within a 176bp region upstream of the *CLB5* open reading frame. Most surprisingly we discovered that the MBF-independent expression of *CLB5* during sporulation critically relies on MCB elements in its promoter (Chapter IV). The essential requirement for MCB sequences within the *CLB5* promoter, which function in the absence of Mbp1 activity, is a profound result that opens a very important door for further investigation of this regulation. This strongly implicates the existence of novel MCB-binding factors encoded within the *S. cerevisiae* genome. In an effort to address this possibility, we made a bold, yet very preliminary attempt to provide evidence for other MCB-binding factors (see Chapter IV). Our demonstration of specific MCB-binding activity in sporulating *mbp1* cells came as a very welcome surprise, because it has set the stage for pursuit of this novel factor. Though we recognized that this endeavor would be a veritable "fishing trip", we did make a further attempt to follow this course. Figure VI-1 presents a preliminary attempt to enrich for the novel MCB-binding activity we observed. Here we treated extracts from sporulating *mbp1* mutants with NH_2SO_4 fractionation, and were able to demonstrate that this activity could be separated within the extract. We realized that with greater enrichment of this activity, it might be possible to isolate the specifically shifted species from the native gel, and with appropriate treatment, identify this factor by mass spectrometry. Unfortunately, we recognized that this assay would require a significant amount of time to determine appropriate conditions to maximize the enriched activity. With time becoming a factor for my graduate program, and with no guarantee of identifying this novel factor, we reasoned that this project would be too risky for further venture. Though I whole-heartedly agreed with this decision, abandoning this search left me rather unsettled. The discovery of another MCB-binding transcription factor in *S. cerevisiae* would have provided a significant contribution towards understanding how MBF functions.

VI.2.c. – Demonstrating Direct Ndt80-Dependence: Investigating the nature of Ndt80-dependent regulation of *CLB5* offered a more suitable closure to my graduate work. Following this direction allowed me to present a more thorough analysis of the *CLB5* promoter, and provided me with enough data to produce a complete study. I also personally feel that these experiments required a significant amount of efficiency and organization on my part, due to the sensitive nature of the techniques employed. Therefore, this study served as an appropriate test of my training within the lab. I feel that the biggest contribution that this section of my thesis makes to the field of cyclin regulation is to demonstrate that Ndt80 "directly" regulates a B-type cyclin during meiotic development in *S. cerevisiae*. Though this was already strongly believed to be the case, to my knowledge, it has not been conclusively determined before now. Therefore, MSE elements recognized within *CLB* promoters are almost definitely authentic Ndt80 binding sites. Indeed, our work has emphasized the importance of confirming these sites. By disproving the previously proposed, strongly consensus MSE

(MSE1) within the *CLB5* promoter, and revealing another MSE sequence (MSE2) which is specifically bound by Ndt80 and conveys Ndt80-dependent expression to this gene, we have demonstrated the importance of promoter analysis. Based on our findings, we present the *CLB5* promoter as being regulated by two MSEs of "unequal" transcriptional potential. Even though I strongly believe that MSE2 serves as the only functional Ndt80 binding site within this sequence, I cannot conclusively make this claim. If I were to suggest one more experiment to put the "nail" in this "coffin", it would be to perform chromatin immunoprecipitation (ChIP) assays on the various MSE promoter constructs we have engineered. If we can determine that Ndt80 only binds to those promoters with wild type MSE2 sequences, and its association does not correlate with MSE1 at all, then we can make a more convincing claim for our proposed Ndt80 binding site. However, by weighing the gains versus the losses, and realistically recognizing the impact of this work, we chose to conclude this study.

VI.3 – Finale

As I emerge from the wake of graduate school, I am satisfied to know that this document will be a standing representation of my effort and accomplishment here at the University of Alberta. It not only summarizes my contributions to science thus far, but also defines my development into a young scientist. I finish my graduate training much more capable then when I began, and with a strong drive to continue my quest for knowledge and scientific skills. Today I will look back to where I've come from, and then look forward to what lies ahead.

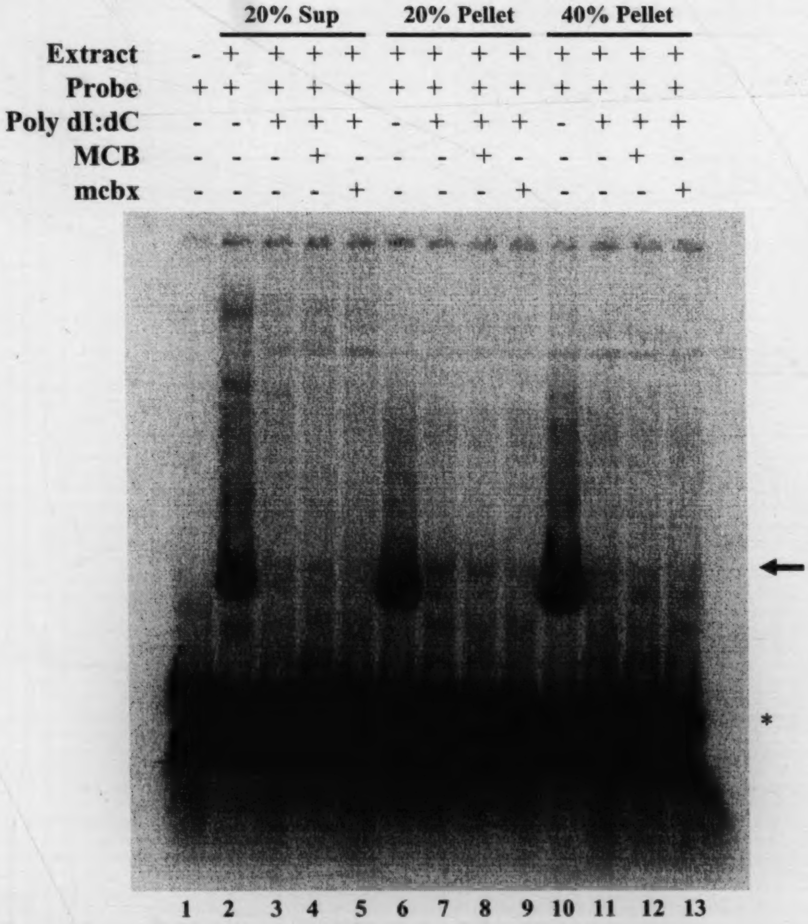

20% Sup
20% Pellet
40% Pellet
Extract - + + + + + + + + + + + +
Probe + + + + + + + + + + + + +
Poly dI:dC - - + + + - + + + - + + +
MCB - - - + - - - + - - - + -
mcbx - - - - + - - - + - - - +
1 2 3 4 5 6 7 8 9 10 11 12 13
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Figure VI-1: Fractionation of *mbp1* extracts with NH_2SO_4 demonstrates the ability to enrich for MCB-binding activity. A crude soluble protein extract from sporulating *mbp1* diploids was split into two equal volumes. One fraction was treated with 20% NH_2SO_4, and the other with 40% NH_2SO_4. Samples from soluble supernatant (Sup) and precipitated pellets (Pellet) were analyzed for MCB-binding activity using EMSA as described in Chapter IV. ^{32}P-radiolabled MCB probe (1pmol) is added to each reaction. All lanes except lane 1 contain 40µg of fractionated *mbp1* extract (20% Sup, lanes 2-5; 20% Pellet, lanes 6-9; 40% Pellet, lanes 10-13). Poly dI:dC (200X) is added to lanes 3-5, lanes 7-9 and lanes 11-13. Cold excess wild type MCB competitor (MCB, 100pmol) is added to lanes 4, 8 and 12; cold excess mutant MCB competitor (mcbx, 100pmol) is added to lanes 5, 9 and 13. A specifically shifted species is indicated with an arrow (←), and unbound labeled probe is indicated with an asterisk.